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2 **STATE-OF-THE-ART IN FAST LIQUID CHROMATOGRAPHY-MASS**  
3 **SPECTROMETRY FOR BIO-ANALYTICAL APPLICATIONS.**

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## 46 Acronyms

47	
48	ADS Alkyl-diol silica
49	AIF All ion fragmentation
50	APCI Atmospheric pressure chemical ionization
51	API Atmospheric pressure ionization
52	APPI Atmospheric pressure photoionization
53	ATCA 2-aminothiazoline-4-carboxylic acid
54	CBA Propylcarboxylic acid
55	ESI Electrospray
56	FDA Food and drug administration
57	FWHM Full width at half maximum
58	HETP height equivalent to a theoretical plate
59	HILIC hydrophilic interaction chromatography
60	HRMS High Resolution Mass Spectrometry
61	HSS High Strength Silica
62	ISRP internal surface reversed-phase

63	IT-TOF	Ion trap time-of-flight
64	LLE	Liquid-Liquid Extraction
65	LOQ	Limit of Quantitation
66	LSD	Lysergic acid diethylamide
67	MIP	Molecular Imprinted Polymers
68	MISBSE	MIPs stir bar sorptive extraction
69	MS	Mass Spectrometry
70	MSn	Multiple stage mass spectrometry
71	NIP	non- imprinted polymer
72	NNAL	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol
73	PE	phosphatidylethanolamines
74	PPT	Protein precipitation
75	PRS	Propylsulphonic acid
76	RAM	Restricted Acces Material
77	RSD	Relative Standard Deviation
78	SBSE	Stir Bar Sorptive Extraction
79	SCID	source collision-induced dissociation
80	SPME	Solid Phase MicroExtraction
81	SRM	Selected reaction monitoring
82	TFC	Turbulent Flow Chromatography
83	UHPLC	Ultra-High Pressure Liquid Chromatography
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94 **Abstract**

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96           There is an increasing need of new bio-analytical methodologies with enough sensitivity,  
97 robustness and resolution to cope with the analysis of a large number of analytes in complex  
98 matrices in short analysis time. For this purpose, all steps included in any bio-analytical method  
99 (sampling, extraction, clean-up, chromatographic analysis and detection) must be taken into  
100 account to achieve good and reliable results with cost-effective methodologies. The purpose of  
101 this review is to describe the state-of-the-art of the most employed technologies in the period  
102 2009-2012 to achieve fast analysis with liquid chromatography coupled to mass spectrometry  
103 (LC-MS) methodologies for bio-analytical applications. Current trends in fast liquid  
104 chromatography involve the use of several column technologies and this review will focus on the  
105 two most frequently applied: sub-2  $\mu\text{m}$  particle size packed columns to achieve ultra high  
106 pressure liquid chromatography (UHPLC) separations and porous-shell particle packed columns  
107 to attain high efficiency separations with reduced column back-pressures.

108           Additionally, recent automated sample extraction and clean-up methodologies to reduce  
109 sample manipulation, variability and total analysis time in bio-analytical applications such as on-  
110 line solid phase extraction coupled to HPLC or UHPLC methods, or the use of other approaches  
111 such as molecularly imprinted polymers, restricted access materials, and turbulent flow  
112 chromatography will also be addressed. The use of mass spectrometry and high or even ultra-high  
113 resolution mass spectrometry to reduce sample manipulation and to solve ion suppression or ion  
114 enhancement and matrix effects will also be presented. The advantages and drawbacks of all  
115 these methodologies for fast and sensitive analysis of biological samples are going to be  
116 discussed by means of relevant applications.

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## 125 **1. Introduction**

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127           The need of high-throughput separations in bio-analytical applications able to cope with  
128 the analysis of a large number of analytes in very different and complex matrices has increased  
129 considerably in the last years. The main objective of any laboratory, including bio-analytical  
130 ones, is to develop reliable and efficient procedures to perform both qualitative and quantitative  
131 analysis with cost-effective methodologies with reduced analysis time. HPLC appears as the most  
132 common approach to solve multiple analytical problems, as it is able to separate quite  
133 complicated mixtures of analytes with different molecular weights as well as different polarities  
134 and acid-base properties. However conventional HPLC alone do not solve all the analytical  
135 problems related to bio-analytical applications and will not always satisfy the need of reducing  
136 the total analysis time in a field with a huge variety of analytes and sample matrices but also with  
137 an increased demand on fast analytical results. Challenges in bio-analytical laboratories include  
138 development of fast LC-MS methods able to separate closely related compounds (e.g. analytes  
139 and metabolites) from endogenous components. Developments in LC-MS and related techniques  
140 has been recently reviewed [1], by giving an overview of recent developments in the last decade  
141 about mass analyzers, ionization techniques, fast LC-MS, LC-MALDI-MS, ion mobility  
142 spectrometry, as well as emerging mass spectrometric approaches complementary to LC-MS.  
143 Additionally, several bio-analytical methods include monitoring of drugs in a variety of  
144 biological matrices in order to evaluate their pharmacokinetics, to establish appropriate dosages,  
145 or to determine drugs, drugs of abuse and their metabolites in forensic analysis. Many of these  
146 methods are required to obtain results very fast in order to take medical, forensic or legal  
147 decisions, and at very low concentration levels because of, for instance, the bioavailability of  
148 many of these drugs, the application of low doses or a fast elimination of the drugs due to  
149 excretion or metabolism, between others. The final objective consists of developing bio-  
150 analytical methods that meets the rigorous criteria set by validation guidelines in terms of  
151 selectivity, accuracy (trueness and precision) and linearity [2], but also guaranteeing  
152 confirmation of target and the identification of related and new compounds [3].

153           Nowadays, there are several approaches in HPLC methods which enable the reduction of  
154 the analysis time without compromising resolution and separation efficiency such as the use of  
155 monolithic columns [4-7] or high temperature liquid chromatography [8-10]. But among them the

156 main approach, including bio-analytical applications, to achieve high-throughput separations is  
157 the use of ultra-high pressure liquid chromatography (UHPLC) using sub-2  $\mu\text{m}$  particle packed  
158 columns [11,12]. Additionally, porous shell columns (packed with sub-3  $\mu\text{m}$  superficially porous  
159 particles) are starting to be used for fast chromatographic separations [13-16].

160 Despite the advances in chromatographic separations techniques, the complexity of  
161 biological sample matrices makes direct analysis by HPLC problematic. For instance, irreversible  
162 adsorption of proteins in the stationary phase can occur, producing loss of column efficiency and  
163 increase in column backpressure. Therefore, the use of ultra-fast separations is not enough to  
164 develop fast analytical methods in bio-analysis, and sample treatment is still one of the most  
165 important parts of the analytical process; effective sample preparation is essential for achieving  
166 good analytical results. Sample preparation has usually been performed using protein  
167 precipitation (PPT), liquid-liquid extraction (LLE) or solid phase extraction (SPE), but these  
168 procedures are in general laborious and time-consuming. An ideal sample preparation method  
169 would be fast, accurate, precise and keep sample integrity. Over the last years, considerable  
170 efforts have been made to develop modern approaches in sample treatment techniques that enable  
171 the reduction of analysis time without compromising the integrity of the extraction process [17].  
172 The use of on-line SPE, which minimizes sample manipulation and provides both high pre-  
173 concentration factors and recoveries, is an increasingly powerful and rapid technique used to  
174 improve the sample throughput and overcome many of the limitations associated with the  
175 classical off-line SPE procedure. Higher specificity and selectivity together with satisfactory  
176 extraction efficiency can be obtained using sorbents based on molecularly imprinted polymers  
177 (MIPs). SPE based on MIPs is a highly attractive and promising approach for matrix clean-up,  
178 enrichment and selective extraction of analytes in such kind of complex samples [18]. The use of  
179 restricted-access materials (RAM) for direct injection of biological samples appears as a good  
180 alternative for selective sample clean-up or fractionation in proteome and peptidome analysis  
181 [19]. Another modern trend in sample preparation for bio-analytical applications is the use of  
182 turbulent-flow chromatography (TFC) that can be even more efficient at removing proteins based  
183 on their size than RAM or SPE [20].

184 The reduction of the analysis time by combining ultra-fast separations and reduced  
185 sample treatments may introduce new analytical challenges during method development. More  
186 matrix related compounds may be introduced into the chromatographic system by reducing

187 sample treatment, and although higher chromatographic resolution and separation efficiency can  
188 be achieved by UHPLC methods, the likelihood of matrix effects during ionization, such as ion  
189 suppression or ion enhancement, may increase. Additionally, the use of on-line SPE procedures  
190 coupled to UHPLC is not a problem-free approach. Conventional on-line SPE systems are not  
191 usually compatible with UHPLC and a loss on the chromatographic efficiency may be observed  
192 when both methodologies are combined. To solve some of these problems the use of liquid  
193 chromatography coupled to mass spectrometry (LC-MS) or tandem mass spectrometry (LC-  
194 MS/MS) is mandatory and for some applications even high resolution mass spectrometry  
195 (HRMS) may be required [21-23].

196 The aim of this review is to discuss the state-of-the-art in fast liquid chromatography  
197 coupled to mass spectrometry and on-line sample preparation techniques for bio-analytical  
198 applications. It should be pointed out that “fast analysis” and “fast chromatography” are terms  
199 currently used without a formal definition. A certain number of compounds analysed per unit  
200 time would be unequivocal analytical parameters. For instance, typical peak widths in routine  
201 UHPLC-MS bio-analyses are 3-10 s [24], while peak widths in the fast/ultrafast LC-MS methods  
202 are generally in the range 1-3 s [25], but they can be narrower than 1 s under well optimized  
203 conditions. However, these parameters are not reported in many cases. In the present review, we  
204 have considered analysis times up to 10 min for both fast chromatography and fast analysis. The  
205 review includes a selection of the most relevant papers recently published (2009-2012) regarding  
206 instrumental and column technology in bio-analysis, particularly UHPLC methods with sub-2  $\mu\text{m}$   
207 and novel porous shell particle packed columns. Modern sample treatment procedures such as on-  
208 line SPE, the use of MIPs and RAM technology, and turbulent-flow chromatography will also be  
209 addressed.

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## 211 **2. Sample preparation**

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### 213 **2.1. On-line solid phase extraction**

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215 Laboratory automation and high-throughput analysis have recently become of primary  
216 importance to reduce analysis time, costs and variability derived from sample manipulation. With  
217 the development of fast chromatographic methods able to separate species in a few minutes with

218 low solvent consumption, it became a priority to shorten conventional sample treatments as well.  
219 In this context, sample preparation using 96-, 384-well or even higher density plate formats is a  
220 well-established technique that has been successfully adopted for semi-automation of off-line  
221 sample procedure in bio-analytical applications [26,27]. 96-well SPE technology and automation  
222 workstations significantly improve the assay sample throughput by reducing both time and labor  
223 required for generating bioanalytical results compared to the conventional off-line SPE methods  
224 [28-31].

225 Another powerful alternative to the classical off-line SPE is represented by the on-line  
226 SPE technology. This technique has revolutionized sample preparation, receiving a lot of interest  
227 in recent years because of its ability to dramatically increase assay performance while reducing  
228 total analysis time. Furthermore, recent developments and advancements in on-line SPE aspects  
229 in combination with the sensitivity and selectivity achieved by MS/MS have made possible the  
230 development of faster and precise on-line SPE-LC- and UHPLC-MS/MS methods for both  
231 qualitative and quantitative analysis of several classes of substances in biological matrices. For  
232 instance, it has shown to be very advantageous for the analysis of steroid hormones, insecticides,  
233 antibacterial, perfluorinated compounds, therapeutic peptides, immunosuppressant,  
234 antidepressant or illicit drugs in a wide range of biological fluids such urine, blood, serum,  
235 plasma, saliva, synovial fluid, milk and other tissues (see Table 1, [32-62]).

236 The comparison of diverse purification and determination techniques provides evidences  
237 to assess the strengths and limitations of on-line SPE compared to other approaches. For instance,  
238 König *et al.* [63] developed an on-line SPE LC-MS/MS method for the determination of the  
239 principal psychoactive constituent of cannabis plant and some of its metabolites in human blood  
240 for use in forensic toxicology as an alternative to their pre-existing method based on GC-MS. The  
241 stationary phase of the trapping and analytical columns were hydrophobic. The on-line method,  
242 which was validated, presented limits of detection in the region of  $1 \mu\text{g L}^{-1}$ . Furthermore, the on-  
243 line SPE permitted overcoming some downsides of the sample treatment stage previous to the  
244 GC-MS analysis such as a laborious sample preparation, long analysis time, and frequent  
245 preventive cleaning of the instrumentation, which is particularly critical with GC-MS. This on-  
246 line SPE approach was also used for the analysis of one of the metabolites in human urine [43].  
247 In this case, no significant matrix effect was observed, excellent intra- and inter-assay precisions  
248 (RSD < 7%) were achieved, with limits of detection in the same range than those observed with



249 the on-line SPE method developed for blood analysis [63]. Carryover was not observed even  
250 though high levels of the studied compounds were injected [63].

251 In a study where LLE, protein precipitation, off-line and on-line SPE were assessed for  
252 the analysis of a cephalosporin in plasma, the first two approaches provided low sensitivity and  
253 interferences by endogenous compounds [64]. The off-line clean-up provided the best sensitivity  
254 and selectivity; however the on-line SPE clean-up offered the shorter analysis time as well as a  
255 lower consumption of reagents and still keeping good sensitivity and selectivity. A compromise  
256 between the methods tested gave the optimal results: off-line protein precipitation followed by  
257 on-line SPE method [64], approach carried out in many of the works quoted in Table 1. Examples  
258 of the advantage of using on-line SPE-LC-MS/MS method in terms of reduction of analysis time  
259 were recently reported for the quantification of free catecholamines in urine [65], where it  
260 allowed to perform their determination in 3% of the time initially spent with sample preparation  
261 and chromatographic separation. Another example of short analysis time is the accurate  
262 determination of 3 triazole antifungal drugs in plasma [37] within 3 minutes. To further reduce  
263 run time together with an additional increase in the detection sensitivity, on-line SPE systems  
264 have also been recently coupled to UHPLC using sub-2  $\mu\text{m}$  particle size columns. For instance,  
265 Ismaiel *et al.* [66] developed a selective UHPLC-MS/MS method for the determination of the  
266 anti-cancer therapeutic peptide ocreotide in human plasma using on-line ion-exchange SPE with  
267 a total run time of 7.5 min and LOQ of 25  $\text{pg mL}^{-1}$ . Moreover, the on-line removal of  
268 phospholipids using column switching and pre-column back-flushing allowed reducing the  
269 matrix effect to less than 4%. The direct hyphenation of on-line SPE to UHPLC system has also  
270 been reported as a powerful analytical tool for microdosing studies in humans for the clinical  
271 development of drug candidates [34]. Furthermore, this study also compared conventional LC-  
272 MS/MS method to UHPLC method; the latter approach leads to 5-fold lower injection volume  
273 and 1.5-fold higher peaks.

274 On-line SPE methods for bio-analysis provide limited purification in the sense that highly  
275 aqueous solvents are used to wash analytes in the trap column. This rinse step is generally not  
276 enough when hydrolysis or precipitation of macromolecules are required because the system  
277 could get block during the pretreatment [55,61,67]. Precisely, system blockage and ion  
278 suppression are some of the reasons that keep the injection volumes relatively low, typically <  
279 200  $\mu\text{l}$  [61], which play against achieving higher preconcentration factors and sensitivity [45]. To

280 isolate the analytes from biological matrices, either straightforward or extensive pretreatment  
281 stages have been applied. Urine samples were just filtered and kept in cool conditions [55];  
282 saliva was diluted and centrifuged [56]; and serum, plasma and brain microdialysate samples  
283 were injected directly onto the on-line SPE and proteins rinsed with a solution with high water  
284 content [39,68]. However, most commonly, precipitation of proteins is carried out off-line with  
285 organic solvents [36,37,52,59], acid [38,40,59,61]; and/or centrifugation [56] or even SPE  
286 [52,69], LLE [39] or purification with an immunoaffinity column [57] prior to injection of an  
287 aliquot of the supernatant into the on-line SPE system. Besides, off-line pretreatment is carried  
288 out to increase the lifetime of the costly columns used for SPE [52]. Two consecutive purification  
289 steps with on-line SPE cartridges prior dilution and centrifugation of saliva samples provided  
290 thorough cleaning and allowed to reuse them 15 times with high precision [56]. The effect of the  
291 clean-up on the instrumental sensitivity was assessed by some authors, for instance, 50 injections  
292 of 400  $\mu$ L of deproteinized plasma into a polymeric SPE cartridge resulted in a two-fold  
293 reduction of the signal in a MS with off-axis ESI [38]. A novel and promising approach for on-  
294 line deproteinization has been carried out with the synthesis of the a polymeric porous monolith  
295 poly(N-isopropylacrylamide-co-ethyleneglycol dimethacrylate), which showed LC-UV  
296 chromatograms with absence of interferences after the direct injection of spiked urine and plasma  
297 [70]. Other approaches to remove macromolecules on-line involving MIPs, RAM and turbulent  
298 flow chromatography will be reviewed in the following sections. Chromatograms obtained with  
299 LC-UV have given an overview on the on-line purification [64,70-74], technique that unlike MS,  
300 also tolerates the presence of phosphate buffers in the mobile phase. When using MS the  
301 purification achieved has been assessed by post-column infusion of the study compound in a  
302 chromatographic run of blank biological sample and observing the reduction of the signal  
303 [56,67,75]; by observing the peak height in absence or presence of matrix [69] or by comparing  
304 the slope of the external calibration curve and standard addition curves [59].

305 Most of the compounds analysed, shown in Table 1, were charged low molecular weight  
306 molecules and their determination was carried out with ESI-MS. Ion exchange sorbents could  
307 potentially provide higher selectivity for the extraction of these analytes than reversed phase  
308 sorbents, which would result in cleaner samples and reduced ion suppression. However, the  
309 studies generally opted for sorbents with hydrophobic interaction with the analytes ( $C_4$ ,  $C_8$ ,  $C_{18}$ ,  
310 polydivinyl-benzene (Hysphere GP resin), N-vinylpyrrolidone–divinylbenzene copolymer

311 (Waters HLB)). A small number of works chose ion-exchange or mixed-mode ion-exchange as  
312 the purification mechanism. Specifically, functionalized silica with propylcarboxylic acid (CBA)  
313 or with propylsulphonic acid (PRS), and polymer based sorbents such as carboxy-  
314 divinylbenzene-N-vinylpyrrolidone co-polymer (Oasis WCX), divinylbenzene-based Bond elute  
315 Plexa PCX or benzenepropanoic acid (Strata X-CW) are among the sorbents most often used in  
316 the works quoted in Table 1 whereas immunosorbents have scarcely been used.

317 The high versatility and purification potential of on-line methodology has been shown in  
318 the fast and quantitative multicomponent analysis in complex biological samples. As an example,  
319 a simple on-line laboratory set-up was automated for simultaneous determination of forty-two  
320 drugs belonging to different chemical classes in human urine within 11 minutes [42]. The sample  
321 clean-up was performed using a SPE Strata X-CW and the separation was performed by UHPLC-  
322 MS/MS. The validation results on linearity, precision, accuracy, matrix and memory effect were  
323 found to be satisfactory, with recovery average greater than 93.8% and LODs/LOQs levels  
324 suitable for confirmation tests.

## 325

## 326 **2.2. Molecularly imprinted polymers (MIPs) and restricted access materials (RAM)**

## 327 **technology**

328

329 The analysis of compounds in biological fluids is a contest between the analytical  
330 demands (best quality parameters and shortest analysis time) and the complexity of the sample.

331 Due to the drawbacks of the commonly used SPE phases, a great effort has been made to  
332 study and develop new sorbents able to increase the overall efficiency of the extraction process  
333 from bio-matrices. These new materials try to accomplish the requirements according to present  
334 needs, such as selectivity towards target analytes, easy manipulation allowing on-line  
335 configurations and higher biocompatibility. Among them, MIPs RAM are currently attracting  
336 much interest.

337 MIPs, also called synthetic antibodies, are polymeric materials possessing an artificially  
338 generated three-dimensional network with highly specific and selective recognition sites [76].  
339 These recognition sites are obtained by polymerizing functional and cross-linking monomers  
340 around a template molecule, followed by subsequent removal of the template in order to leave a  
341 cavity with binding sites complementary to the shape, size and functional groups of the target

342 compound [77]. This technology has grown in popularity over the past few years compared to  
343 other techniques such as conventional SPE or immunoaffinity sorbents because of the advantages  
344 of being at the same time highly selective, cost-effective, and not suffering from storage  
345 limitations and stability problems associated with organic solvents or extreme pH values. An  
346 example of the superior features of MIPs when compared to traditional SPE has been recently  
347 reported for the extraction of an illicit drug such as lysergic acid diethylamide (LSD) from hair  
348 and urine samples [78]. MIP was used for off-line extraction before LC-MS analysis and its  
349 performance compared to that of a conventional C<sub>18</sub> SPE. Molecularly imprinted SPE showed  
350 higher recoveries (~83%) than commercially C<sub>18</sub> SPE (~65%) with a significant improvement in  
351 analytical sensitivity. Thus, because of the potential benefits of using this technique, MIP-SPE  
352 coupled to LC-MS has been extensively applied for the selective extraction and pre-concentration  
353 of a wide range of analytes, such as benzodiazepines [79], zidovudine and stavudine from human  
354 plasma [80], cocaine and its metabolite benzoylecgonine [81], ketamine and norketamine [82] in  
355 hair samples, as well as testosterone, epitestosterone [83], and 4-(methylnitrosamino)-1-(3-  
356 pyridyl)-1-butanol from urine samples [84]. However, even if the use of MIP particles as  
357 selective sorbents for solid-phase extraction (MIP-SPE) is by far the most common application  
358 of MIPs, molecularly imprinted polymers have also been used with satisfactory results as coating  
359 agents for stir bar sorptive extraction (SBSE) and solid-phase microextraction (SPME) fibers, or  
360 as stationary phase for capillary micro-columns. For instance, a high-throughput on-line  
361 microfluidic sample extraction method using capillary micro-columns packed with MIP beads  
362 coupled with tandem mass spectrometry was reported for the analysis of urinary 4-  
363 (Methylnitrosamino)-1-(3-pyridyl)-1-butanol [85]. The developed method, which has been  
364 validated according to Food and Drug Administration (FDA) guideline on bio-analytical method  
365 validation [2], has a short run time of 7 min and requires the use of small sample volumes (200  
366 µl), reaching limits of quantitation as low as 20 pg mL<sup>-1</sup>. MIPs, as coating agents for stir bar  
367 sorptive extraction (MISBSE), were also developed for determination of 2-aminothiazoline-4-  
368 carboxylic acid (ATCA) as a marker for cyanide exposure in forensic urine analysis [86]. The  
369 performance of this column-less method, based on MISBSE combined with LC-MS/MS, was  
370 demonstrably adequate for the analysis of ATCA at pg µL<sup>-1</sup> levels without the use of any  
371 derivatization step. Furthermore, MS/MS was used to improve the overall selectivity of the  
372 method and overcome problems associated with matrix interferences due to the possible co-

373 extraction of other urinary acids by the MISBSE procedure. Finally, a somewhat different  
374 approach was recently successfully used for the determination of antibiotics drugs in human  
375 plasma as well as in simulated body fluids [87]. In this study, MIPs were applied as an alternative  
376 for selective SPME coating. MIP-coated fibers for SPME were prepared by using electrochemical  
377 polymerization of pyrrole and linezolid as template molecules. The developed SPME MIP-coated  
378 fibers were then applied to the determination of selected antibiotic drugs such as linezolid,  
379 daptomycin and amoxicillin. The method is shown to be rapid, reproducible and with a detection  
380 limit for linezolid of 29 ng mL<sup>-1</sup>. Furthermore, the selectivity of the SPME MIP-coated fibers for  
381 these antibiotic drugs was assessed by comparing its activity with the non-imprinted polymer  
382 (NIP)-coated fibers. As expected, SPME MIP-coated fibers showed higher binding capacity  
383 compared with NIPs. In summary, MIPs appears as a very useful and promising approach for  
384 sample extraction and clean-up procedure in bio-analytical applications, where a very high degree  
385 of selectivity may be required to reduce analysis time without suffering from problems related to  
386 sensitivity of MS detection such as matrix effects. RAMs in automated sample preparation  
387 systems on-line coupled with sensitive techniques such as LC-MS/MS or LC-fluorescence have  
388 been an effective strategy to overcome that analytical challenge [88-90]. The use of RAMs  
389 simplifies the purification of low molecular weight substances in bio-fluids by physical and  
390 chemical diffusion barrier. RAMs have a dual surface configuration; the outer surface employs  
391 both size exclusion and hydrophilic shielding to create a non-adsorptive outer surface of the  
392 particles and prevent macromolecules accessing the inner surfaces where smaller molecules can  
393 be adsorbed by hydrophobic interaction.

394 The RAMs used today are derived from the first sorbents developed in 1985 [91], called  
395 internal surface reversed-phase (ISRP) materials. Alkyl-diol silica materials (ADS) [92] are  
396 among the RAM sorbents most frequently used today and are commercially available. ADS have  
397 a bimodal function based on diol groups in the outer part and hydrophobic extraction phases (C4,  
398 C8 or C18) in the interior. For instance, they have been applied for the analysis of vitamin D and  
399 metabolites from serum [93], mercapturic acids in urine [94,95] or multiresidue analysis of  
400 xenobiotics in urine [96]. But aside from these well-established sorbents, important steps forward  
401 in the development of new RAM sorbents have taken place as it is following described.

402 The incorporation of restricted access properties to magnetic particles has opened the door  
403 to a new modality of sample preparation supports for bio-analysis. Magnetic porous silica

404 microspheres were synthesised through polymerization-induced silica/magnetite colloid  
405 aggregation and calcination. The microspheres were subsequently modified with alkyl groups on  
406 the internal surface and diol groups on the external surface [97]. Another magnetic RAM,  
407 schematized in Figure 1, was prepared by functionalizing magnetite nanoparticles with  
408 dodecyltriethoxysilane and non-ionic surfactant (tween). The extraction efficiency was tested for  
409 the analysis of estrogens in urine. Salting out effect was found to increase the extraction  
410 efficiency, pH was not found to be a critical factor for this application and the addition of an  
411 organic modifier reduced their performance [98]. The properties of RAM and MIP materials have  
412 merged in the RAM-MIP grafted silica synthesized by Wenjuan Xu et al. [99], who by a  
413 controlled polymerization technique (see method in [100]) prepared an advanced material  
414 consisting of an internal polymer imprinted with sulfonamides and an hydrophilic external layer  
415 of glycerol monomethacrylate that prevented the adsorption of proteins. This advanced RAM has  
416 been successfully used in the extraction and clean-up of sulphonamides from milk [99].

417         Novelties in the uses of RAM to solve bio-analytical problems have also taken place. The  
418 purification of a protein from the family of cytokines, of about 20 KDa, from plasma with RAM  
419 has been carried out despite these sorbents are generally used for retaining low molecular weight  
420 compounds. The RAM used in this case was constituted by silica particles with bonded C18 and  
421 serum albumin [101]. The optimization of the coupling of a RAM (MSPak) with N-  
422 vinylacetamide copolymer as stationary phase with a hydrophilic interaction chromatography  
423 (HILIC) column for the analysis of nucleosides in urine, overcoming the compatibility problems  
424 between the solvent required for the elution of the RAM the mobile phase used in the separation,  
425 represented a step forward in this field of sample preparation [102].

426         Reusing RAM sorbents after being loaded with biological samples which underwent a  
427 minor or no sample treatment [94-96,101,102] proves the efficiency of the technique and shows  
428 its superiority to the single use of SPE cartridges. However, despite the advantageous features of  
429 RAMs, these materials still have limitations. The elution of the analytes from the RAM to the  
430 analytical column is a key step in the purification process; the sorbent could still contain residues  
431 of macromolecules at this point if the washing step has not been fully effective. To avoid the  
432 precipitation of residual macromolecules in the RAM, the amount of organic solvents in the  
433 transference is kept low, usually below 15%, but a disadvantage of the weak eluotropic strength  
434 of the transfer solution is that residual amounts of the analytes can remain in the sorbent and

435 cause false positives. Carryover may be likely to be happen when injecting a hydrophobic analyte  
436 in a reversed phase RAM. For instance, carryover of bosentan and metabolites, which are  
437 compounds with 4 aromatic cycles, was assessed to happen at about 0.2% of the last injected  
438 sample [103]. The assessment of carryover can be carried out directly with the analysis of blanks  
439 [96,101,103] or indirectly with the assessment of the recovery through the RAM [102], recovery  
440 of the whole analysis including sample treatment, separation and detection [101] or analysis of  
441 reference materials [93].

442

### 443 **2.3. Turbulent flow chromatography**

444

445 Turbulent flow chromatography is widely used in applications where plasma or similar  
446 fluids are to be analyzed. This technique allows the direct injection of a liquid sample onto a  
447 narrow diameter column (0.5 or 1.0 mm) packed with large particles (30-60  $\mu\text{m}$ ) at a high flow  
448 rate (higher than 1  $\text{mL min}^{-1}$ ). Under turbulent flow conditions, there is improved mass transfer  
449 across the bulk mobile phase which allows improving the radial distribution of the analytes.  
450 However, under these conditions, a laminar zone where diffusional forces still dominate the mass  
451 transfer process still exists around the stationary phase [104]. Molecules with low molecular  
452 weight diffuse faster than molecules with high molecular weight, forcing large molecules to  
453 quickly flow to waste while retaining the small ones. The retained compounds are then back-  
454 flushed and focused on the analytical column for chromatographic separation, like in an on-line  
455 extraction with RAM. The first application of TFC-MS for the direct injection of plasma was  
456 described in 1997 by Ayrton *et al.* [105]. Many more studies have been reported in successive  
457 years applied to various matrices covering from biological (Table 2, [104,106-118]) to  
458 environmental and food samples. For example, the successful analysis of immunosuppressants  
459 and antibiotics from low volume samples such as ocular fluid (tears) and whole blood has been  
460 reported [106,119,120]. Besides, this technology has been applied to the analysis of more  
461 complex matrices such as hemodialysates [121], edible animal tissues [122] and food samples  
462 such as honey [123] and milk [124]. The major advantage of TFC in comparison to other  
463 extraction techniques is the reduction of time consuming preparation steps while similar LOQ,  
464 dynamic range, accuracy and precision can be obtained. The main drawback of this technique is  
465 probably the low concentration capacity achieved although this can be compensated by the use of

466 capillary LC leading to the reduction of the amount of sample volume injected [125]. In addition,  
467 this technique is clearly limited in terms of chromatographic resolution. Therefore, it is common  
468 to couple turbulent flow columns to a more conventional analytical column by means of column  
469 switching systems. TFC is considered to be similar to SPE followed by liquid chromatography  
470 although the extraction in TFC is size-exclusion based, therefore it is mainly applied when there  
471 is an interest to separate small analyte molecules from larger matrix molecules [125]; the larger  
472 molecules, such as proteins, go directly to waste and the smaller molecules are adsorbed to the  
473 retentive stationary phase of the turbulent flow column. Nevertheless, TFC seems to be more  
474 efficient at removing proteins than RAM or SPE [126]. In general its simplicity, versatility and  
475 automation possibilities are well described in the literature as it dramatically increases the speed  
476 of the analysis while maintaining acceptable levels of recovery, efficiency and robustness. The  
477 analysis time can vary between 2-9 minutes depending on the number of compounds and the  
478 number of channels used (multiplexing). Mueller *et al.* described a reduction of 50% on the  
479 analysis of sirolimus and everolimus by using TFC, nevertheless the analysis time reported (5.75  
480 minutes) is longer in comparison to other on-line methods [106]. The authors adopted a slightly  
481 longer analysis time in a way to have better robustness, no carryover and extended column  
482 lifetime (>600 injections) provided by extending cleaning and equilibration times. On the other  
483 hand, real screening methods including a large number of analytes have also been reported where  
484 the analysis time can be extended to up 32 minutes [107]. In this case, more than 400 compounds  
485 are included in the acquisition list. Off-line handling of the sample is often limited to  
486 centrifugation, for removal of particulates, dilution with internal standard and protein  
487 precipitation (PPT) to remove endogenous binding proteins [109]. The latter helps preventing  
488 column clogging.

489         Mueller *et al.* [107] reported a comprehensive toxicological MS<sup>n</sup> screening method for the  
490 analysis of serum and heparinised plasma. This methodology targeted 453 compounds and the  
491 results were cross-checked with urine samples to test the performance of the method under  
492 realistic clinical conditions as well as to compare the information gathered using different  
493 matrices. Pérez *et al.* [108] applied the same principles for bioaccumulation studies of  
494 perfluorinated compounds in human hair and urine. Similar results were obtained between  
495 radioimmunoassay and TFC by Bunch *et al.* [109] in the analysis of 25-hydroxyvitamin D2 and  
496 D3 in serum.



497 The sample preparation technique used has clear effects on the composition of the  
498 purified extract. In the case of metabonomic studies, an automated sample preparation  
499 methodology in which the sample can be injected directly into the system is appealing.  
500 Michopoulos *et al.* [104] reported that even though the use of TFC in metabonomic studies is  
501 feasible, different profiles were obtained when comparing TFC and protein precipitation. This  
502 was attributed to the greater amount of phospholipids in the protein-precipitated samples, due to  
503 fact that TFC would not affect the binding of such compounds to proteins; they would pass  
504 through the first column without being retained [104].

505 The robustness of sample preparation techniques in quantitative assays is crucial.  
506 Methods using PPT are simple and cheap but produce relatively dirty extracts which may reduce  
507 the lifetime of the chromatographic column, extend the cleaning/maintenance of the mass  
508 spectrometer or result in matrix effects [127]. LLE and SPE are effective in terms of producing  
509 clean extracts and reducing matrix effect but are also laborious and difficult to automate.

510 TFC can also be used for analytical support of *in vivo* pharmacokinetics and *in vitro* drug  
511 metabolism studies [113,128]. Verdirame *et al.* [113] reported the advantage of TFC  
512 methodologies in terms of sensitivity and throughput in comparison to conventional procedures.  
513 In this case, a set up consisting of two parallel turbulent flow and two analytical columns  
514 operating independently was used. This configuration allowed a 4-fold improvement in terms of  
515 throughput.

516 Summarizing, the optimization of the different on-line extraction steps is crucial, as  
517 parameters like mobile phase composition, flow rates and extraction time windows will affect  
518 recovery and extraction efficiency. In general, TFC provides simplicity, automation, robustness,  
519 versatility and high-throughput in bio-analysis.

520

### 521 **3. Trends in chromatography approaches**

522

#### 523 **3.1. Ultrahigh pressure liquid chromatography**

524

525 Fast chromatography has become a reality in laboratories that require analyzing hundreds  
526 of samples per day or those needing short turnaround times. The development of columns packed  
527 with sub-2  $\mu\text{m}$  particles and the commercialization of LC systems capable of withstanding

528 pressures as high as 1000 bar lead to a significant increase in the analysis throughput. By using  
529 UHPLC, the results of a sample batch can be reported in a few hours rather than a few days as it  
530 is needed, for instance, in doping control laboratories. Thus, the demand of high sample  
531 throughput in short time frames have given rise to high efficiency and fast liquid  
532 chromatographic separations in several fields, including bio-analysis, using mainly reversed-  
533 phase columns packed with sub-2  $\mu\text{m}$  particles. Moreover, this column technology for UHPLC  
534 emerged as a powerful approach particularly because of the ability to transfer existing HPLC  
535 conditions directly [129].

536 In general, fast chromatographic separations can be achieved either by increasing the  
537 mobile phase flow-rate, by decreasing the column length or by reducing the column particle  
538 diameter. Based on the van Deemter theory [130], then on Giddings [131], and later on Knox  
539 [132] and further interpretations, the efficiency, expressed as the height equivalent to a theoretical  
540 plate (HETP,  $H$ ), can be described as:

541

$$542 \quad H = A + B/u + Cu = 2\lambda d_p + 2\gamma D_M / u + f(k)d_p^2 u / D_M$$

543

544 where  $u$  is the linear velocity of the mobile phase, and  $A$ ,  $B$ , and  $C$  are constants related to Eddy  
545 diffusion, longitudinal diffusion and mass transfer in mobile and stationary phase, respectively,  
546  $d_p$  is the particle diameter of the column packing material,  $D_M$  is the analyte diffusion coefficient,  
547  $\lambda$  is the structure factor of the packing material,  $\gamma$  is a constant termed tortuosity or obstruction  
548 factor and  $k$  is the retention factor for a given analyte [130]. So basically, HETP depends on three  
549 terms, which are the band broadening due to Eddy diffusion coefficient ( $A$ -term), longitudinal  
550 diffusion coefficient ( $B$ -term) and the resistance to mass transfer coefficient between the mobile  
551 and stationary phases ( $C$ -term). It is often assumed that  $A$ -term does not depend on temperature  
552 and it is directly proportional to  $d_p$ , while  $B$ - and  $C$ -terms are both temperature dependent, the  $B$ -  
553 term being directly proportional to  $D_M$  while the  $C$ -term is inversely proportional to  $D_M$  but  
554 directly proportional to the square of  $d_p$ . So, as lower is the particle diameter of the column  
555 packing material higher will be the column efficiency (lower HETP), and high throughput  
556 separations will be achieved. The use of small particles will induce a considerable increase in  
557 pressure drop, but this inconvenience has been resolved with the availability of new ultra-high

558 pressure resistant LC systems allowing to profit fully from the advantages in using sub-2  $\mu\text{m}$   
559 particle packed columns.

560 The narrow peaks that will be produced by fast UHPLC separations will require detection  
561 systems with a small detection volume and fast acquisition rates in order to keep the high  
562 efficiency gained in the separation. Most of the commercial UHPLC instruments available are  
563 equipped with modified UV detectors, with flow cell volumes much lower than those for  
564 conventional HPLC, in order to ensure the optimal peak capture. However, due the complexity of  
565 sample matrices such as biological samples, UHPLC couple to mass spectrometry has become  
566 the method of choice in bio-analytical applications in order to guarantee the confirmation of  
567 target compounds. Moreover, because UHPLC enhances chromatographic resolution overall, co-  
568 elution is reduced, and that, in turn, leads to a decrease of ion suppression, improving MS  
569 sensitivity and reliability. However, since UHPLC greatly enhances separation throughput and  
570 resolution, base peaks as narrow as 1 s (or even lower) can be obtained creating practical issues  
571 for bio-analytical applications. MS instruments are required to work at low dwell times and low  
572 inter-channel and inter-scan delays in order to achieve a sufficient amount of data points (e.g., >  
573 15 points per peak) for UHPLC methods to ensure reliable quantitation [24].

574 Several recent applications of UHPLC-MS methods in bio-analysis using sub-2  $\mu\text{m}$   
575 particle size packed columns are summarized in Table 3 [42,58,59,66,133-157]. As can be seen,  
576 most of the UHPLC applications using columns packed with sub-2  $\mu\text{m}$  particles are focused in  
577 the analysis of mainly plasma (or blood related matrices) [58,59,66,133-144,150,151,153,155-  
578 157] and urine [42,138,139,142,146-150] matrices, although applications in other biological  
579 samples such as several tissues [153,154], tumor tissues [152,153], faeces [150], human seminal  
580 plasma [145], and saliva [142] have also been reported. For instance, Baumgarten *et al.* [152]  
581 developed an UHPLC-MS/MS method using a C18 column packed with 1.6  $\mu\text{m}$  particles for the  
582 rapid confirmation of doxorubicin drug delivery in liver cancer tissue after a transcatheter arterial  
583 chemoembolization treatment used for palliative therapy. The method allowed the separation of  
584 doxorubicin and daunorubicin within 1 min and helped in the better understanding of the factors  
585 affecting the delivery and dispersion of doxorubicin within treated tumors during these  
586 treatments. McWhinney *et al.* [142] developed an UHPLC-MS/MS method for the laboratory  
587 routine analysis of glucocorticoid hormones in several matrices such as plasma, plasma  
588 ultrafiltrate, urine and saliva. As an example, Figure 2 shows the chromatographic separation of

589 cortisol, cortisone, 11-deoxycortisol, prednisolone and dexamethasone hormones (Figure 2a) as  
590 well as the chromatograms obtained after application of the proposed UHPLC-MS/MS method to  
591 the analysis of several matrices (Figures 2b-2f). Chromatographic separation of all  
592 glucocorticoids in less than 2.5 min was achieved showing limits of quantitation in the range of 1  
593 to 5 nmol L<sup>-1</sup> (depending on the sample matrix) and with intra-assay and inter-assay precisions  
594 with RSD values lower than 5 and 10%, respectively, for all compounds in all matrices.

595 Most of the applications are based on reversed-phase separation using the Acquity UPLC  
596 BEH C18 column of 1.7 µm particle size with different columns lengths (30, 50 or 100 mm), but  
597 other C18 reversed-phase columns such as Zorbax Eclipse XDB-C18 (1.8 µm particle size)  
598 [42,59,136,139,150], Shimadzu Shim-pack ODS (1.6 µm particle size) [152] or Hypersil Gold  
599 C18 (1.9 µm particle size) [58,144,148,151,157] have also been used. Although not strictly sub-2  
600 µm particle size columns, some bio-analytical UHPLC-MS applications can be found using  
601 columns with slightly higher totally porous particle sizes. As an example, Tuffal *et al.* [140]  
602 reported the use of a Shimadzu Shim-pack XR-ODS II column of 2.2 µm particle size for the  
603 UHPLC-MS separation of clopidogrel active metabolite isomers in plasma in less than 7 min.  
604 Other stationary phases have also been described for UHPLC-MS bio-analytical applications. For  
605 instance, the use of a high strength silica (HSS) column (Acquity UPLC HSS T3, 1.8 µm particle  
606 size) was reported by Vanden Bussche *et al.* [149] for the analysis of eight thyreostats in urine,  
607 without any derivatisation, in less than 6.5 min. While Jiménez Giron *et al.* [146] used a C8  
608 reversed-phase column (Zorbax SB-C8, 1.8 µm particle size) for the UHPLC-High resolution  
609 Orbitrap MS screening analysis of diuretic and stimulant compounds in urine for doping control.  
610 By screening in full scan MS with scan-to-scan polarity switching more than 120 target analytes  
611 could be detected in less than 8 min.

612 Regarding MS detection, triple quadrupole analyzers are instruments of choice for  
613 UHPLC-MS bio-analytical applications as can be seen in Table 3. Other MS and HRMS  
614 instruments have also been used for some bio-analytical applications, which will be discussed in  
615 further detail in section 4.

616

### 617 **3.2. Fused-core particle packed columns**

618

619 The recent commercialization of fused-core (also known as porous shell) particle  
620 technology presents a new option for HPLC bio-analytical applications in order to achieve fast  
621 chromatographic and high efficiency separations. Today, columns packed with porous shell  
622 particles consisting of silica particles of a 1.7  $\mu\text{m}$  fused core and 0.5  $\mu\text{m}$  layer of porous silica  
623 coating, creating a total particle diameter of 2.7  $\mu\text{m}$ , are available under the brand name HALO  
624 (Advance Materials Technology) or Ascentis (Sigma-Aldrich). Other particle diameter sizes are  
625 also available such as in the case of Kinetex (Phenomenex) columns with a 1.9  $\mu\text{m}$  fused core  
626 and 0.35  $\mu\text{m}$  layer of porous silica coating, obtaining 2.6  $\mu\text{m}$  particles and Accucore (Thermo  
627 Fisher Scientific) columns with also a total particle diameter of 2.6  $\mu\text{m}$ . This fused-core column  
628 technology, with a solid silica inner core surrounded by a porous silica shell has a shortened  
629 diffusion path which allows rapid mass transfer and thus reduced axial dispersion and peak  
630 broadening [158]. The reduction in axial diffusion makes possible working at higher flow-  
631 without losing chromatographic performance [159]. So, fused-core silica particles offer the  
632 possibility to improve chromatographic column efficiency over fully porous particles, and exhibit  
633 efficiencies that are comparable to sub-2  $\mu\text{m}$  porous particles, but with lower backpressures. For  
634 instance, Figure 3 shows the chromatographic separation of bromo-guanosine, labetalol, reserpine  
635 and a selected drug compound obtained with two conventional particle size columns (Luna  
636 C18(2) HST 2.5  $\mu\text{m}$  and Luna PFP 3  $\mu\text{m}$ ), a sub-2  $\mu\text{m}$  particle size column (Acquity BEH C18  
637 1.7  $\mu\text{m}$ ) and a fused-core column (Ascentis Espresso C18 2.7  $\mu\text{m}$ ) [160]. The fused-core column  
638 showed similar peak widths than the other columns (similar column efficiency) at approximately  
639 75% of the maximum specified backpressure for this column, even after more than 1500  
640 injections of protein precipitated plasma extracts. It should be noted that the most popular  
641 UHPLC stationary phase material, Acquity BEH C18 1.7  $\mu\text{m}$  column, operates at high  
642 backpressure (>700 bars) even with the column oven set at 65  $^{\circ}\text{C}$  (combined with an efficient  
643 mobile phase pre-heating device in-line prior to the UHPLC column). Higher pressures were  
644 obtained when the column oven temperature was set at 40  $^{\circ}\text{C}$ , leading to concern regarding the  
645 robustness of the system for application in the successful conduct of thousands of analyses of  
646 extracted plasma samples.

647 The use of porous shell column technology is a relatively recent trend in chromatographic  
648 separations and only a few papers about bio-analytical applications are described in the literature,  
649 and some of the most recent ones have been included in Table 4 [160-165]. As can be seen, all

650 the applications are dealing with C18 reversed-phase separations. As in the case of columns  
651 packed with sub-2  $\mu\text{m}$  particles, triple quadrupole instruments are usually selected for UHPLC-  
652 MS applications. For instance, Song *et al.* [165] proposed an UHPLC-MS/MS method using a 2.7  
653  $\mu\text{m}$  fused-core column and a triple quadrupole instrument for the analysis of imipramine and  
654 desipramine antidepressants in protein precipitated rat plasma samples, and a separation within  
655 2.5 min was achieved at a flow rate of  $0.4\text{ mL min}^{-1}$ , with acceptable intra-run precisions and  
656 accuracies (within 14.4 and 14.7% at the LOQ level for both analytes). However, other MS  
657 instruments such as quadrupole linear ion traps for the analysis of oseltamivir and  
658 oseltamivircarboxylate in dried blood spots [163], or even high resolution mass spectrometry  
659 using a TOF MS instrument for the UHPLC-MS analysis of isoliquiritigenin metabolites in urine  
660 [162] or a linear ion trap-Orbitrap HRMS instrument for the analysis of glutathione-trapped  
661 reactive metabolites in plasma [161] have also been reported.

662

#### 663 **4. Mass spectrometry in bio-analysis**

664

665 LC-MS has proven to be a powerful technique in bio-analysis. ESI and atmospheric  
666 pressure chemical ionization (APCI) are the most common ionization sources used in LC-MS.  
667 Nevertheless, ESI operating in both negative and positive modes is in general the most selected  
668 ionization source in bio-analytical science (Table 3 and 4). However, many studies have reported  
669 difficulties with reproducibility and accuracy when analyzing small quantities of analytes in  
670 complex samples such as biological fluids. Because of the specificity of the MS methods,  
671 analysis times in LC-MS assays are often reduced significantly by researchers, due to the  
672 misconception that chromatographic separation and sample preparation can be minimized or even  
673 eliminated. However, LC-MS by itself does not guarantee selectivity. Disregarding sample  
674 clean-up, especially when complex matrices are involved, will lead to poor performance. Thus,  
675 careful consideration must be given to the evaluation and the elimination matrix effects when  
676 developing any assay. Ion suppression is one the major problems in LC-MS with atmospheric  
677 pressure ionization (API) sources, especially with ESI. Ion suppression occurs due to the  
678 competition among several ions during ion evaporation [166]. Nowadays, as it is reported in this  
679 review, fast liquid chromatography and fast sample analysis are commonly used to reduce the  
680 analysis time and determine the maximum number of compounds in the same run. However,

681 important matrix effects can be present and its evaluation is necessary to obtain accurate  
682 quantitation results. Generally, in order to reduce matrix effects, one strategy can be to improve  
683 the sample preparation procedure which is in conflict with the fast and non-selective sample  
684 treatment procedures that are demanded. Recent breakthroughs in sample clean-up have been  
685 achieved to exploit on-line SPE and TFC with column switching systems, where matrix  
686 components are diverted to waste before the elution step, hence the amount of undesirable  
687 compounds reaching the LC-MS system is reduced. Another way to reduce the matrix effect is to  
688 optimize the chromatographic separation and increase the chromatographic resolution. In this  
689 way, and as it was commented in the section 3.1 and 3.2 the use of sub-2  $\mu\text{m}$  particle size  
690 columns and porous shell columns is increasing. Nevertheless, important matrix effects can still  
691 be observed and the use of alternative ionization sources less sensitive to matrix effects such as  
692 APCI and atmospheric pressure photoionization (APPI) is proposed. For instance, Mueller *et al.*  
693 [106] developed a TFC-LC-MS/MS method using APCI as ionization source for the analysis of  
694 sirolimus and its derivative everolimus, two immunosuppressive agents, in whole blood in order  
695 to reduce the matrix effects observed when ESI was used. APCI ionization source has been also  
696 used for the multi-screening of drugs and metabolites in serum and plasma [107,114], as well as  
697 for the analysis of vitamins [109] and tyrosinekinase inhibitors [111]. On the other hand, some  
698 results indicate that APPI is less susceptible to ion suppression and salt-buffer effects than ESI  
699 and APCI [167]. For instance, Borges *et al.* [48] developed a LC-APPI-MS/MS method for the  
700 analysis of ethinylestradiol in human plasma. In this study the use of APPI provided better  
701 sensitivity than ESI and in addition no significant matrix effects were observed making possible  
702 the analysis of ethinylestradiol at low concentration levels. On the other hand, this ionization  
703 technique has been successfully used for the analysis of a broad spectrum of non-polar lipids  
704 such as steroids, (glycol-)sphingolipids, and phytosterols [168]. However, until now there are  
705 only few publications regarding the use of APPI in bio-analysis and much work needs to be done  
706 to evaluate its potential in this area.

707 Triple quadrupole instruments operating in selected reaction monitoring (SRM) mode are  
708 the most common analyzers used in bio-analysis (Table 3 and 4). As a compromise between  
709 sensitivity, acceptable chromatographic peak shape, and the confirmation purposes established by  
710 2002/657/EC directive [3] two SRM transitions are currently monitored. However, in some cases  
711 the use of only two transitions could result in false-positive or false-negative confirmations when

712 the compound co-elutes with an interfering matrix compound with ions in the MS/MS spectrum  
713 matching with those of the target analyte [169-171]. In these cases, false-positive results can be  
714 prevented with by further confirmatory analysis, e.g. the use of a third transition or an orthogonal  
715 criterion like exact mass measurements. On the other hand, despite its high selectivity and  
716 sensitivity the use of SRM acquisition mode in QqQ instruments is limited by the cycle time  
717 when dealing with hundreds of compounds, and a significant drawback to this type of analyzers  
718 is that only those molecules that have been targeted are detected (missing non-target compounds  
719 or even target metabolites). For these reasons, nowadays to solve the problems related to both the  
720 cycle time and the target screening method, liquid chromatography coupled to high resolution  
721 mass spectrometry (LC-HRMS) is being implemented in bio-analysis. Time-of-flight (TOF) and  
722 Orbitrap based technologies are currently the most common analyzers used in LC-HRMS. For  
723 instance Fung *et al.* [172] proposed a LC-HRMS method using a quadrupole-time-of-flight (Q-  
724 TOF) analyzer for the analysis of prednisone and prednisolone in human plasma operating at a  
725 mass resolving power of 10,000 and obtaining mass errors below 6 ppm. On the other hand,  
726 Jiménez Girón *et al.* [146] reported a new screening method based on UHPLC-HRMS using  
727 polarity switching for the analysis of 122 targeted analytes in urine by direct analysis. In this  
728 work the use of polarity switching acquisition mode in combination with HRMS allowed the  
729 possibility to obtain two diagnostic ions. This strategy was used to confirm some diuretics  
730 compounds that exhibit high sensitivity in negative mode but were also detectable in positive  
731 mode. The use of high resolution mass spectrometry is also especially useful in metabolomics  
732 studies where full scan MS spectra and accurate mass measurements are acquired for  
733 identification purposes [173]. The use of ultra-high resolution mass spectrometry, operating at  
734 mass resolving power higher than 30,000 FWHM is especially important in lipidomics studies  
735 due to the complexity of this family of compounds. Taking advantage of the ultra-high resolution  
736 provided by an Orbitrap analyser isobaric phosphatidylethanolamines (PE) ether species with 7  
737 double bonds (which are common in several model organisms, such as *C. elegans*) could be  
738 differentiated from the PE ester species with two saturated fatty acid moieties having a mass  
739 difference of 0.0575 Da (Figure 4, [174]). However, in this case operating at ultra high resolution  
740 mass spectrometry has compromised the scan speed and therefore the coupling to fast LC  
741 separation.



742           However, in some cases the unequivocal identification of target and target-related  
743 compounds requires combining the information provided by HRMS and MS/MS experiments.  
744 Moreover, accurate mass measurements and elemental composition assignment are essential for  
745 the characterization of small molecules. For instance, the accurate mass measurements of the  
746 product ions generated in MS<sup>n</sup> experiments facilitate the elucidation of unknown compounds  
747 structures, making attractive the use of hybrid mass spectrometers such as Q-TOF, ion-trap -  
748 time-of-flight, linear ion-trap quadrupole – Orbitrap (LTQ-Orbitrap) and quadrupole-Orbitrap (Q-  
749 Exactive). In this way, a new concept in tandem mass spectrometry, “all ion fragmentation”  
750 (AIF) experiments, have been recently introduced. This acquisition mode enables the  
751 combination of the fragmentation of all generated ions entering into the collision cell with the full  
752 scan MS data, allowing retrospective data evaluation for unknown substances in any untargeted  
753 approach, and consequently providing an extra confirmation strategy. AIF acquisition mode has  
754 become highly important in some bio-analytical applications such as in doping control. For  
755 instance, Thomas *et al.* [157] developed a LC-HRMS(/MS) method for the analysis of some  
756 prohibited drugs in dried blood spots for doping control with AIF acquisition mode. This strategy  
757 was also followed by Zhu *et al.* [161] in the screening of glutathione-trapped metabolites in  
758 human plasma where AIF, non-selective in-source collision-induced dissociation (SCID)  
759 fragmentation and HRMS were used. Study in which the putative metabolites could be confirmed  
760 and their structures elucidated with the corresponding high resolution full scan and high  
761 resolution MS/MS data acquired using a LTQ-Orbitrap velos instrument.

762

## 763 **Conclusions and future perspectives**

764

765           Fast or ultra-fast separation methods appear as a good tool to satisfy the necessity of  
766 reducing the total analysis time in bio-analysis where a high number and variety of samples are  
767 being analyzed, and in areas where results must be reported promptly. The state-of-the-art of fast  
768 LC-MS for bio-analytical applications have been discussed in this review. Nowadays, UHPLC  
769 technology is the most convenient approach to achieve modern, high throughput, efficient,  
770 economic and fast LC separations for bio-analytical applications using sub-2 $\mu$ m particle size  
771 packed columns. Although different stationary phases i.e. reversed phase, hydrophilic interaction  
772 liquid chromatography (HILIC), fluorinated columns, etc. are available in sub-2 $\mu$ m particle size

773 columns, most of the bio-analytical applications are still focused mainly in C18 or C8 reversed-  
774 phase columns (Table 3). The use of HILIC is becoming very popular for bio-analytical  
775 applications allowing better separation of highly polar compounds than with reversed-phase  
776 chromatography and it will become a complementary tool to explore in the near future for  
777 UHPLC bio-analytical applications. Although separation with columns packed with sub-2 $\mu$ m  
778 particles requires special instrumentation because of the high pressures achieved, instruments  
779 adapted to operate at these pressures are commercially available. However, this drawback can  
780 also be compensated with the use of porous shell columns, which can be employed in any HPLC  
781 or UHPLC instrument because the fused-core particle design allows to considerably reducing  
782 column backpressure but keeping similar column efficiency than what is achieved in sub-2 $\mu$ m  
783 particle size columns. Columns packed with porous shell particles seems to be a more  
784 advantageous approach to easily achieve fast LC separations even when using conventional LC  
785 instrumentation, and it will also become a field to explore in the next years for bio-analytical  
786 applications as an alternative to sub-2 $\mu$ m particle size columns.

787         Despite the important advances in fast liquid chromatography able to separate species in a  
788 few minutes with low solvent consumption, sample extraction and clean-up treatments must be  
789 carefully developed to reduce total analysis time. The most recently introduced sample treatment  
790 automated methodologies in bio-analytical applications have also been addressed in this review,  
791 such as on-line SPE, TFC, MIP and RAM based methods. It should be pointed out that sample  
792 preparation techniques must be chosen and optimized on the basis of the method purpose and  
793 take into consideration the chromatographic separation that will be used. In this context, recent  
794 developments in on-line SPE aspects in combination with the sensitivity and selectivity achieved  
795 by MS/MS have made possible the development of faster and precise on-line SPE-LC- and  
796 UHPLC-MS/MS methods for both qualitative and quantitative analysis of heterogeneous  
797 substances in biological matrices.

798         TFC appears as a very useful approach for sample treatment in bio-analytical applications  
799 where plasma or similar fluids need to be analyzed by removing proteins based on their size  
800 better than restricted access materials or SPE procedures. For these reasons, TFC is one of the  
801 modern approaches in sample treatment procedures that is becoming more popular and a number  
802 of applications are expected to be available in the future beyond the bio-analytical field.

803 MIPs and RAM have attracted much interest in the last years (Table 1). The use of MIP is  
804 a very useful approach for some bio-analytical applications because it makes possible to achieve  
805 a selective preconcentration of the target analytes. One of the main advantages of MIPs is the  
806 possibility to prepare selective sorbents pre-determined for a particular substance or a group of  
807 structural analogs, which will become very useful for some specific applications. On-line RAM  
808 (together with TFC) are solvent-less techniques and, although using mobile phase for sample  
809 elution, are among the most environmentally friendly sample treatment procedures. And although  
810 some drawbacks exist when using RAM such as the risk of carryover when the sample contains  
811 hydrophobic species and the method involves reversed-phase RAM and transfer solution with low  
812 elutropic strength, advances are taking place in the development of new RAM sorbents. For  
813 instance, the incorporation of restricted access properties to magnetic particles are providing  
814 supports for new and relevant bio-analytical applications.

815 Regarding mass spectrometry, the use of triple quadrupole instruments monitoring two  
816 SRM transitions is the most common approach used for bio-analytical applications. Nevertheless,  
817 in several cases the use of only two transitions resulted in false-positive or false-negative  
818 confirmations. Moreover, one of the major drawbacks in bio-analysis when using QqQ analyzers  
819 is that only targeted molecules are being detected, missing important information for some bio-  
820 analytical applications. Nowadays, HRMS, either using TOF or Orbitrap analyzers, is being  
821 implemented in bio-analytical analysis to solve these problems. The possibility of working at  
822 high resolving power together with accurate mass measurements makes these instruments ideal to  
823 facilitate identification of unknown compounds which is essential for some bio-analytical  
824 applications. In this way, Orbitrap instruments working in AIF mode will become a powerful tool  
825 for bio-analytical applications in the future.

826 Comprehensive analysis and testing is needed to evaluate these methodologies applied  
827 into bio-analytical applications. It is necessary that all steps in analytical method development,  
828 sample treatment (extraction and clean-up), chromatographic separation and detection, are  
829 developed and optimized in alignment, focusing in the reduction of the total analysis time in  
830 order to achieve fast methods but without compromising the reliability and quality of the  
831 analytical results, especially in a field with direct implications for human health such as bio-  
832 analysis.

833

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## References

- [1] M. Holcapek, R. Jirasko, M. Lisa, *J. Chromatogr. A* 1259 (2012) 3.
- [2] International Conference on Harmonization (ICH) Guidelines, Q2(R1): Validation of Analytical Procedures: Text and Methodology, US FDA Federal Register, November 2005. Available: [http://www.ich.org/fileadmin/Public\\_Web\\_Site/ICH\\_Products/Guidelines/Quality/Q2\\_R1/Step4/Q2\\_R1\\_Guideline.pdf](http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1_Guideline.pdf)
- [3] European Commission 2002/657/EC, Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. European Commission, Brussels.
- [4] O. Núñez, K. Nakanishi, N. Tanaka, *J. Chromatogr. A* 1191 (2008) 231.
- [5] C. Heideloff, D.R. Bunch, S. Wang, *Ther. Drug Monit.* 32 (2010) 102.
- [6] A. Kadi, M. Hefnawy, A. Al Majed, S. Alonezi, Y. Asiri, S. Attia, E. Abourashed, H. El Subbagh, *Analyst* 136 (2011) 591.
- [7] H. Du, J. Ren, S. Wang, *Food Chem.* 129 (2011) 1320.
- [8] T. Teutenberg, *Anal. Chim. Acta* 643 (2009) 1.
- [9] S. Heinisch, J.L. Rocca, *J. Chromatogr. A* 1216 (2009) 642.
- [10] J.M. Cunliffe, J.X. Shen, X. Wei, D.P. Dreyer, R.N. Hayes, R.P. Clement, *Bioanalysis* 3 (2011) 735.
- [11] N. Wu, A.M. Clausen, *J. Sep. Sci.* 30 (2007) 1167.
- [12] G. D'Orazio, A. Rocco, S. Fanali, *J. Chromatogr. A* 1228 (2012) 213.
- [13] F. Gritti, A. Cavazzini, N. Marchetti, G. Guiochon, *J. Chromatogr. A* 1157 (2007) 289.
- [14] S. Fekete, J. Fekete, K. Ganzler, *J. Pharm. Biomed. Anal.* 49 (2009) 64.
- [15] S. Fekete, K. Ganzler, J. Fekete, *J. Pharm. Biomed. Anal.* 54 (2011) 482.
- [16] S. Fekete, J. Fekete, *Talanta* 84 (2011) 416.
- [17] P.L. Kole, G. Venkatesh, J. Kotecha, R. Sheshala, *Biomed. Chromatogr.* 25 (2011) 199.
- [18] A. Beltran, R.M. Marce, P.A.G. Cormack, F. Borrull, *J. Chromatogr. A* 1216 (2009) 2248.

- 869 [19] N.M. Cassiano, J.C. Barreiro, M.C. Moraes, R.V. Oliveira, Q.B. Cass, *Bioanalysis* 1  
870 (2009) 577.
- 871 [20] Josep L. Herman, Molecular Weight Exclusion of Proteins Using Turbulent Flow  
872 Chromatography, Poster n. 72, 27th Montreux Symposium on LC/MS, Montreux,  
873 November 2010, Switzerland.
- 874 [21] W.B. Emary, N.R. Zhang, *Bioanalysis* 3 (2011) 2485.
- 875 [22] K. Scheffler, E. Damoc, M. Kellmann, *GIT Labor-Fachz.* 55 (2011) 516.
- 876 [23] W. Korfmacher, *Bioanalysis* 3 (2011) 1169.
- 877 [24] D. Guillarme, J. Schappler, S. Rudaz, J.L. Veuthey, *TrAC, Trends Anal. Chem.* 29 (2010)  
878 15.
- 879 [25] E. Uliyanchenko, P.J. Schoenmakers, S. van der Wal, *J. Chromatogr. A* 1218 (2011)  
880 1509.
- 881 [26] R.N. Xu, L. Fan, M.J. Rieser, T.A. El-Shourbagy, *J. Pharm. Biomed. Anal.* 44 (2007)  
882 342.
- 883 [27] M.S. Chang, E.J. Kim, T.A. El-Shourbagy, *Rapid Commun. Mass Spectrom.* 21 (2007)  
884 64.
- 885 [28] K. Ardjomand-Woelkart, M. Kollroser, L. Li, H. Derendorf, V. Butterweck, R. Bauer,  
886 *Anal. Bioanal. Chem.* 400 (2011) 2371.
- 887 [29] S. Bouchet, E. Chauzit, D. Ducint, N. Castaing, M. Canal-Raffin, N. Moore, K. Titier, M.  
888 Molimard, *Clin. Chim. Acta* 412 (2011) 1060.
- 889 [30] N.K. Jayatilaka, M. Angela Montesano, R.D. Jr Whitehead, S.J. Schloth, L.L. Needham,  
890 D.B. Barr, *Arch. Environ. Contam. Toxicol.* 61 (2011) 59.
- 891 [31] K. Dhillon, T. Ho, P. Rich, D. Xu, F. Lorey, J. She, A. Bhandal, *Clin. Chim. Acta* 412  
892 (2011) 2076.
- 893 [32] C. Emotte, F. Deglave, O. Heudi, F. Picard, O. Kretz, *J. Pharm. Biomed. Anal.* 58 (2012)  
894 102.
- 895 [33] M. Ivanova, C. Artusi, G. Polo, M. Zaninotto, M. Plebani, *Clin. Chem. Lab. Med.* 49  
896 (2011) 1151.
- 897 [34] K. Heinig, T. Wirz, F. Bucheli, V. Monin, A. Gloge, *J. Pharm. Biomed. Anal.* 54 (2011)  
898 742.
- 899 [35] A.L. Saber, *Talanta* 78 (2009) 295.

- 900 [36] J. Shentu, et al., Determination of amlodipine in human plasma using automated online  
901 solid-phase extraction HPLC–tandem mass spectrometry: Application to a bioequivalence  
902 study of Chinese volunteers, *J. Pharm. Biomed. Anal.* (2012),  
903 <http://dx.doi.org/10.1016/j.jpba.2012.06.014>
- 904 [37] K.Y. Beste, O. Burkhardt, V. Kaefer, *Clin. Chim. Acta* 413 (2012) 240.
- 905 [38] W.H. Kwok, D.K.K. Leung, G.N.W. Leung, T.S.M. Wan, C.H.F. Wong, J.K.Y. Wong, *J.*  
906 *Chromatogr. A* 1217 (2010) 3289.
- 907 [39] J. Stevens, D.-J. van den Berg, S. de Ridder, H.A.G. Niederländer, P.H. van der Graaf, M.  
908 Danhof, E.C.M. de Lange, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 878  
909 (2010) 969.
- 910 [40] C. Emotte, O. Heudi, F. Deglave, A. Bonvie, L. Masson, F. Picard, A. Chaturvedi, T.  
911 Majumdar, A. Agarwal, R. Woessner, O. Kretz, *J. Chromatogr. B: Anal. Technol.*  
912 *Biomed. Life Sci.* 895–896 (2012) 1.
- 913 [41] C. Köhler, T. Grobosch, T. Binscheck, *Anal. Bioanal. Chem.* 400 (2011) 17.
- 914 [42] U. Chiuminatto, F. Gosetti, P. Dossetto, E. Mazzucco, D. Zampieri, E. Robotti, M.C.  
915 Gennaro, E. Marengo, *Anal. Chem.* 82 (2010) 5636.
- 916 [43] M.d.M.R. Fernandez, S.M.R. Wille, N. Samyn, M. Wood, M. Lopez-Rivadulla, G. De  
917 Boeck, *J. Chromatogr. , B: Anal. Technol. Biomed. Life Sci.* 877 (2009) 2153.
- 918 [44] Q.Q. Wang, S.S. Xiang, Y.B. Jia, L. Ou, F. Chen, H.F. Song, Q. Liang, D. Ju, *J.*  
919 *Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 878 (2010) 1893.
- 920 [45] D.R. Dufield, O.V. Nemirovskiy, M.G. Jennings, M.D. Tortorella, A.M. Malfait, W.R.  
921 Mathews, *Anal. Biochem.* 406 (2010) 113.
- 922 [46] Z. Leon-Gonzalez, C. Ferreira-Vera, F. Priego-Capote, M.D. Luque de Castro, *J.*  
923 *Chromatogr. A* 1218 (2011) 3013.
- 924 [47] W.H.A. de Jong, R. Smit, S.J.L. Bakker, E.G.E. de Vries, I.P. Kema, *J. Chromatogr. , B:*  
925 *Anal. Technol. Biomed. Life Sci.* 877 (2009) 603.
- 926 [48] N.C. Borges, R.B. Astigarraga, C.E. Sverdloff, P.R. Galvinas, W. Moreira da Silva, V.M.  
927 Rezende, R.A. Moreno, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 877 (2009)  
928 3601.
- 929 [49] C.W. Hu, Y.J. Huang, Y.J. Li, M.R. Chao, *Clin. Chim. Acta* 411 (2010) 1218.
- 930 [50] C. Ferreira-Vera, J.M. Mata-Granados, F. Priego-Capote, J.M. Quesada-Gomez, M.D.  
931 Luque de Castro, *Anal. Bioanal. Chem.* 399 (2011) 1093.
- 932 [51] W.H.A. de Jong, M.H.L.I. Wilkens, E.G.E. de Vries, I.P. Kema, *Anal. Bioanal. Chem.*  
933 396 (2010) 2609.

- 934 [52] D. Thibeault, N. Caron, R. Djiana, R. Kremer, D. Blank, J. Chromatogr. B: Anal.  
935 Technol. Biomed. Life Sci. 883–884 (2012) 120.
- 936 [53] A. Saba, A. Raffaelli, A. Cupisti, A. Petri, C. Marcocci, P. Salvadori, J. Mass Spectrom.  
937 44 (2009) 541.
- 938 [54] F. Kirchhoff, S. Lorenzl, M. Vogeser, Clin. Chem. Lab. Med. 48 (2010) 1647.
- 939 [55] L.C. Lin, S.L. Wang, Y.C. Chang, P.C. Huang, J.T. Cheng, P.H. Su, P.C. Liao,  
940 Chemosphere 83 (2011) 1192.
- 941 [56] R.L. Jones, L.J. Owen, J.E. Adaway, B.G. Keevil, J. Chromatogr. B: Anal. Technol.  
942 Biomed. Life Sci. 881–882 (2012) 42.
- 943 [57] C.J. Wang, N.H. Yang, S.H. Liou, H.L. Lee, Talanta 82 (2010) 1434.
- 944 [58] H.T. Liao, C.J. Hsieh, S.Y. Chiang, M.H. Lin, P.C. Chen, K.Y. Wu, J. Chromatogr. B:  
945 Anal. Technol. Biomed. Life Sci. 879 (2011) 1961.
- 946 [59] F. Gosetti, U. Chiuminatto, D. Zampieri, E. Mazzucco, E. Robotti, G. Calabrese, M.C.  
947 Gennaro, E. Marengo, J. Chromatogr. A 1217 (2010) 7864.
- 948 [60] K. Kato, B.J. Basden, L.L. Needham, A.M. Calafat, J. Chromatogr. A 1218 (2011) 2133.
- 949 [61] C. Mosch, M. Kiranoglu, H. Fromme, W. Völkel, J. Chromatogr. B: Anal. Technol.  
950 Biomed. Life Sci. 878 (2010) 2652.
- 951 [62] D. Kloos, R.J.E. Derks, M. Wijtmans, H. Lingeman, O.A. Mayboroda, A.M. Deelder,  
952 W.M.A. Niessen, M. Giera, J. Chromatogr. A 1232 (2012) 19.
- 953 [63] S. Konig, B. Aebi, S. Lanz, M. Gasser, W. Weinmann, Anal. Bioanal. Chem. 400 (2011)  
954 9.
- 955 [64] J. Li, L. Wang, Z. Chen, R. Xie, Y. Li, T. Hang, G. Fan, J. Chromatogr. B: Anal. Technol.  
956 Biomed. Life Sci. 895–896 (2012) 83.
- 957 [65] W.H.A. de Jong, E.G.E. de Vries, B.H.R. Wolffenbuttel, I.P. Kema, J. Chromatogr. , B:  
958 Anal. Technol. Biomed. Life Sci. 878 (2010) 1506.
- 959 [66] O.A. Ismaiel, T. Zhang, R. Jenkins, H.T. Karnes, J. Chromatogr. B: Anal. Technol.  
960 Biomed. Life Sci. 879 (2011) 2081.
- 961 [67] S. Sturm, F. Hammann, J. Drewe, H.H. Maurer, A. Scholer, J. Chromatogr. B: Anal.  
962 Technol. Biomed. Life Sci. 878 (2010) 2726.
- 963 [68] Y.K. Liu, X.Y. Jia, X. Liu, Z.Q. Zhang, Talanta 82 (2010) 1212.
- 964 [69] U. Lövgren, S. Johansson, L.S. Jensen, C. Ekström, A. Carlshaf, J. Pharm. Biomed. Anal.  
965 53 (2010) 537.



- 966 [70] H. Liu, Y. Duan, Y. Jia, Y. Gu, J. Li, C. Yan, G. Yang, *J. Chromatogr. B: Anal. Technol.*  
967 *Biomed. Life Sci.* 889–890 (2012) 55.
- 968 [71] M.F. El Shahat, N. Burham, S.M.A. Azeem, *J. Hazard. Mater.* 177 (2010) 1054.
- 969 [72] P. Severino, H. Silva, E.B. Souto, M.H. Santana, T.C. Dalla Costa, *J. Pharm. Anal.* 2  
970 (2012) 29.
- 971 [73] T.D. Karakosta, P.D. Tzanavaras, D.G. Themelis, *Talanta* 88 (2012) 561.
- 972 [74] L.E. Vera-Avila, B.P. Márquez-Lira, M. Villanueva, R. Covarrubias, G. Zelada, V.  
973 Thibert, *Talanta* 88 (2012) 553.
- 974 [75] M.M. Ramírez-Fernández, S.M.R. Wille, V. di Fazio, M. Gosselin, N. Samyn, *J.*  
975 *Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 878 (2010) 1616.
- 976 [76] P. Lucci, D. Derrien, F. Alix, C. Perollier, S. Bayoudh, *Anal. Chim. Acta* 672 (2010) 15.
- 977 [77] P. Lucci, O. Nunez, M.T. Galceran, *J. Chromatogr. A* 1218 (2011) 4828.
- 978 [78] F. Chapuis-Hugon, M. Cruz-Vera, R. Savane, W.H. Ali, M. Valcarcel, M. Deveaux, V.  
979 Pichon, *J. Sep. Sci.* 32 (2009) 3301.
- 980 [79] E.C. Figueiredo, R. Sparrapan, G.B. Sanvido, M.G. Santos, M.A. Zezzi Arruda, M.N.  
981 Eberlin, *Analyst* 136 (2011) 3753.
- 982 [80] S.V. Duy, I. Lefebvre-Tournier, V. Pichon, F. Hugon-Chapuis, J.Y. Puy, C. Perigaud, *J.*  
983 *Chromatogr. , B: Anal. Technol. Biomed. Life Sci.* 877 (2009) 1101.
- 984 [81] V. Thibert, P. Legeay, F. Chapuis-Hugon, V. Pichon, *Talanta* 88 (2012) 412.
- 985 [82] N. Harun, R.A. Anderson, P.A.G. Cormack, *Anal. Bioanal. Chem.* 396 (2010) 2449.
- 986 [83] B.T.S. Bui, F. Merlier, K. Haupt, *Anal. Chem.* 82 (2010) 4420.
- 987 [84] H. Hou, X. Zhang, Y. Tian, G. Tang, Y. Liu, Q. Hu, *J. Pharm. Biomed. Anal.* 63 (2012)  
988 17.
- 989 [85] K.A. Shah, M.C. Peoples, M.S. Halquist, S.C. Rutan, H.T. Karnes, *J. Pharm. Biomed.*  
990 *Anal.* 54 (2011) 368.
- 991 [86] R. Jackson, I. Petrikovics, E.P.C. Lai, J.C.C. Yu, *Anal. Methods* 2 (2010) 552.
- 992 [87] M. Szultka, J. Szeliga, M. Jackowski, B. Buszewski, *Anal. Bioanal. Chem.* 403 (2012)  
993 785.
- 994 [88] P. Lucci, D. Pacetti, O. Núñez, N.G. Frega (2012). Current trends in sample treatment  
995 techniques for environmental and food analysis, in *Chromatography*, L. Calderon (Ed.),  
996 InTech, Rijeka, Croatia. ISBN 979-953-307-912-6

- 997 [89] K. Pyrzynska (2011). Solid-Phase Extraction for Enrichment and Separation of  
998 Herbicides, in *Herbicides, Theory and Applications*, S. Soloneski and M.L. Larramendy  
999 (Eds), InTech, Rijeka, Croatia. ISBN: 978-953-307-975-2.
- 1000 [90] N. Fontanals, R.M. Marce, F. Borrull, P.A.G. Cormack, *TrAC, Trends Anal. Chem.* 29  
1001 (2010) 765.
- 1002 [91] I.H. Hagestam, T.C. Pinkerton, *J. Chromatogr. A* 368 (1986) 77.
- 1003 [92] K.S. Boos, A. Rudolphi, S. Vielhauer, A. Walfort, D. Lubda, F. Eisenbeiß, *Fresenius' J.*  
1004 *Anal. Chem.* 352 (1995) 684-690.
- 1005 [93] Baecher S, et al, Simultaneous quantification of four vitamin D metabolites in human  
1006 serum using high performance liquid chromatography tandem mass spectrometry for  
1007 vitamin D profiling, *Clin Biochem* (2012), doi:10.1016/j.clinbiochem.2012.06.030
- 1008 [94] E. Eckert, G. Leng, W. Gries, T. Göen, *J. Chromatogr. B: Anal. Technol. Biomed. Life*  
1009 *Sci.* 889–890 (2012) 69.
- 1010 [95] T. Schettgen, et al., Accurate quantification of the mercapturic acids of acrylonitrile and  
1011 its genotoxic metabolite cyanoethylene-epoxide..., *Talanta* (2012),  
1012 <http://dx.doi.org/10.1016/j.talanta.2012.06.074>.
- 1013 [96] E. Rodríguez-Gonzalo, D. García-Gómez, R. Carabias-Martínez, *J. Chromatogr. A* 1217  
1014 (2010) 40.
- 1015 [97] Y. Wang, Y. Wang, L. Chen, Q.H. Wan, *J. Magn. Magn. Mat.* 324 (2012) 410.
- 1016 [98] L. Ye, Q. Wang, J. Xu, Z.g. Shi, L. Xu, *J. Chromatogr. A* 1244 (2012) 46.
- 1017 [99] W. Xu, S. Su, P. Jiang, H. Wang, X. Dong, M. Zhang, *J. Chromatogr. A* 1217 (2010)  
1018 7198.
- 1019 [100] J. Oxelbark, C. Legido-Quigley, C.S.A. Aureliano, M.M. Titirici, E. Schillinger, B.  
1020 Sellergren, J. Courtois, K. Irgum, L. Dambies, P.A.G. Cormack, D.C. Sherrington, E. De  
1021 Lorenzi, *J. Chromatogr. A* 1160 (2007) 215.
- 1022 [101] A.R. Chaves, B.J.G. Silva, F.M. Lanças, M.E. Queiroz, *J. Chromatogr. A* 1218 (2011)  
1023 3376.
- 1024 [102] E. Rodríguez-Gonzalo, D. García-Gómez, R. Carabias-Martínez, *J. Chromatogr. A* 1218  
1025 (2011) 9055.
- 1026 [103] N. Ganz, M. Singrasa, L. Nicolas, M. Gutierrez, J. Dingemanse, W. Döbelin, M. Glinski,  
1027 *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 885–886 (2012) 50.
- 1028 [104] F. Michopoulos, A.M. Edge, G. Theodoridis, I.D. Wilson, *J. Sep. Sci.* 33 (2010) 1472.

- 1029 [105] J. Ayrton, G.J. Dear, W.J. Leavens, D.N. Mallett, R.S. Plumb, *Rapid Commun. Mass Spectrom.* 11 (1997) 1953.  
1030
- 1031 [106] D.M. Mueller, K.M. Rentsch, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 878  
1032 (2010) 1007.
- 1033 [107] D.M. Mueller, K.M. Rentsch, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 883-  
1034 884 (2012) 189.
- 1035 [108] F. Perez, M. Llorca, M. Farre, D. Barcelo, *Anal. Bioanal. Chem.* 402 (2012) 2369.
- 1036 [109] D.R. Bunch, A.Y. Miller, S. Wang, *Clin. Chem. Lab. Med.* 47 (2009) 1565.
- 1037 [110] D.R. Bunch, C. Heideloff, J.C. Ritchie, S. Wang, *J. Chromatogr. B: Anal. Technol.*  
1038 *Biomed. Life Sci.* 878 (2010) 3255.
- 1039 [111] L. Couchman, M. Birch, R. Ireland, A. Corrigan, S. Wickramasinghe, D. Josephs, J.  
1040 Spicer, R.J. Flanagan, *Anal. Bioanal. Chem.* 403 (2012) 1685.
- 1041 [112] R. Harlan, W. Clarke, J.M. Di Bussolo, M. Kozak, J. Straseski, D. Li Meany, *Clin. Chim.*  
1042 *Acta* 411 (2010) 1728.
- 1043 [113] M. Verdirame, M. Veneziano, A. Alfieri, A. Di Marco, E. Monteagudo, F. Bonelli, J.  
1044 *Pharm. Biomed. Anal.* 51 (2010) 834.
- 1045 [114] D.M. Mueller, B. Duretz, F.A. Espourteille, K.M. Rentsch, *Anal. Bioanal. Chem.* 400  
1046 (2011) 89.
- 1047 [115] A.R. Breaud, R. Harlan, J.M. Di Bussolo, G.A. McMillin, W. Clarke, *Clin. Chim. Acta*  
1048 411 (2010) 825.
- 1049 [116] X. He, M. Kozak, *Anal. Bioanal. Chem.* 402 (2012) 3003.
- 1050 [117] G.Z. Xin, J.L. Zhou, L.W. Qi, C.Y. Li, P. Liu, H.J. Li, X.d. Wen, P. Li, *J. Chromatogr. B:*  
1051 *Anal. Technol. Biomed. Life Sci.* 878 (2010) 435.
- 1052 [118] C. Yuan, C. Heideloff, M. Kozak, S. Wang, *Clin. Chem. Lab. Med.* 50 (2012) 95.
- 1053 [119] P. Mora, U. Ceglarek, F. Manzotti, L. Zavota, A. Carta, R. Aldigeri, J.G. Orsoni, *Graefes*  
1054 *Arch. Clin. Exp. Ophthalmol.* 246 (2008) 1047.
- 1055 [120] A. Hartmann, R. Krebber, G. Daube, K. Hartmann, *J. Vet. Pharmacol. Ther.* 31 (2008) 87.
- 1056 [121] W. Zeng, D.G. Musson, A.L. Fisher, L. Chen, M.S. Schwartz, E.J. Woolf, A.Q. Wang, *J.*  
1057 *Pharm. Biomed. Anal.* 46 (2008) 534.
- 1058 [122] R. Krebber, F.J. Hoffend, F. Ruttman, *Anal. Chim. Acta* 637 (2009) 208.
- 1059 [123] P. Mottier, Y. Hammel, E. Gremaud, P. Guy, *J. Agric. Food Chem.* 56 (2008) 35.

- 1060 [124] A.A.M. Stolker, R.J.B. Peters, R. Zuiderent, J.M. Di Bussolo, C.P.B. Martins, *Anal.*  
1061 *Bioanal. Chem.* 397 (2010) 2841.
- 1062 [125] *Handbook of Analytical Separations, Bioanalytical Separations – Volume 4 – Edited by*  
1063 *Ian Wilson. Published by Elsevier Science B.V (2003).*
- 1064 [126] O. Núñez, H. Gallart-Ayala, C.P.B. Martins, P. Lucci, *J. Chromatogr. A* 1228 (2012) 298.
- 1065 [127] L. Couchman, *Biomed. Chromatogr.* 26 (2012) 892.
- 1066 [128] P.J. Rudewicz, *Bioanalysis* 3 (2011) 1663.
- 1067 [129] L. Novakova, J.L. Veuthey, D. Guillarme, *J. Chromatogr. A* 1218 (2011) 7971.
- 1068 [130] J.J. Van Deemter, F.J. Zuiderweg, A. Klingengerg, *J. Chem. Eng. Sci.* 5 (1956) 272.
- 1069 [131] J.C. Giddings, *Anal. Chem.* 37 (1965) 60.
- 1070 [132] J.H. Knox, *J. Chromatogr. Sci.* 15 (1977) 352.
- 1071 [133] R.W. Sparidans, S. Durmus, N. Xu, A.H. Schinkel, J.H.M. Schellens, J.H. Beijnen, J.  
1072 *Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 895-896 (2012) 174.
- 1073 [134] G. Liu, H.M. Snapp, Q.C. Ji, M.E. Arnold, *Anal. Chem.* 81 (2009) 9225.
- 1074 [135] S. Ding, I. Schoenmakers, K. Jones, A. Koulman, A. Prentice, D.A. Volmer, *Anal.*  
1075 *Bioanal. Chem.* 398 (2010) 779.
- 1076 [136] Y. Gu, W.R. Wilson, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 877 (2009)  
1077 3181.
- 1078 [137] A.M. Evans, C.D. De Haven, T. Barrett, M. Mitchell, E. Milgram, *Anal. Chem.* 81 (2009)  
1079 6656.
- 1080 [138] H. Vlckova, M. Rabatinova, A. Miksova, G. Kolouchova, S. Micuda, P. Solich, L.  
1081 Novakova, *Talanta* 90 (2012) 22.
- 1082 [139] W.d. Zhang, W.j. Yang, X.j. Wang, Y. Gu, R. Wang, *J. Chromatogr. B: Anal. Technol.*  
1083 *Biomed. Life Sci.* 879 (2011) 3735.
- 1084 [140] G. Tuffal, S. Roy, M. Lavisse, D. Brasseur, J. Schofield, N.D. Touchard, P. Savi, N.  
1085 Bremond, M.C. Rouchon, F. Hurbin, E. Sultan, *Thromb. Haemostasis* 105 (2011) 696.
- 1086 [141] C.L. Bowen, J. Kehler, C.A. Evans, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*  
1087 878 (2010) 3125.
- 1088 [142] B.C. McWhinney, S.E. Briscoe, J.P.J. Ungerer, C.J. Pretorius, *J. Chromatogr. , B: Anal.*  
1089 *Technol. Biomed. Life Sci.* 878 (2010) 2863.

- 1090 [143] A. Radovnikovic, M. Moloney, P. Byrne, M. Danaher, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 879 (2011) 159.  
1091
- 1092 [144] Y.W. You, C.E. Uboh, L.R. Soma, F.Y. Guan, X.Q. Li, Y. Liu, J.W. Chen, D. Tsang,  
1093 *Chromatographia* 72 (2010) 1097.
- 1094 [145] A.A. Amoako, T.H. Marczylo, P.M.W. Lam, J.M. Willets, A. Derry, J. Elson, J.C. Konje,  
1095 *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 878 (2010) 3231.
- 1096 [146] A. Jimenez Giron, K. Deventer, K. Roels, P. Van Eenoo, *Anal. Chim. Acta* 721 (2012)  
1097 137.
- 1098 [147] C.H.F. Wong, D.K.K. Leung, F.P.W. Tang, J.K.Y. Wong, N.H. Yu, T.S.M. Wan, J.  
1099 *Chromatogr. A* 1232 (2012) 257.
- 1100 [148] S. Magiera, I. Baranowska, J. Kusa, *Talanta* 89 (2012) 47.
- 1101 [149] J. Vanden Bussche, L. Vanhaecke, Y. Deceuninck, K. Verheyden, K. Wille, K. Bekaert,  
1102 B. Le Bizec, H.F. De Brabander, *J. Chromatogr. A* 1217 (2010) 4285.
- 1103 [150] B. Prasad, S. Singh, *J. Pharm. Biomed. Anal.* 52 (2010) 377.
- 1104 [151] S.J. Mayatra, B. Prasad, M. Jain, R. Jain, S. Singh, *J. Pharm. Biomed. Anal.* 52 (2010)  
1105 410.
- 1106 [152] S. Baumgarten, R.C. Gaba, R.B. van Breemen, *Biomed. Chromatogr.* 26 (2012) 1529.
- 1107 [153] A. Estella-Hermoso de Mendoza, M.A. Campanero, F. Mollinedo, M.J. Blanco-Prieto, J.  
1108 *Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 877 (2009) 4035.
- 1109 [154] R.R. Gonzalez, R.F. Fernandez, J.L.M. Vidal, A.G. Frenich, M.L.G. Perez, *J. Neurosci.*  
1110 *Methods* 198 (2011) 187.
- 1111 [155] H. Vlckova, D. Solichova, M. Blaha, P. Solich, L. Novakova, *J. Pharm. Biomed. Anal.* 55  
1112 (2011) 301.
- 1113 [156] W.J. Kong, X.H. Xia, J.B. Wang, C.P. Zhou, F. Fang, X.Y. Xing, C. Jin, Y.L. Zhao, Q.C.  
1114 Zang, X.H. Xiao, *J. Sep. Sci.* 34 (2011) 260.
- 1115 [157] A. Thomas, H. Geyer, W. Schaenzer, C. Crone, M. Kellmann, T. Moehring, M. Thevis,  
1116 *Anal. Bioanal. Chem.* 403 (2012) 1279.
- 1117 [158] J.J. Kirkland, T.J. Langlois, J.J. DeStefano, *Am. Lab.* 39 (2007) 18.
- 1118 [159] G. Guiochon, *J. Chromatogr. A* 1126 (2006) 6.
- 1119 [160] D.N. Mallett, C. Ramirez-Molina, *J. Pharm. Biomed. Anal.* 49 (2009) 100.
- 1120 [161] X. Zhu, N. Kalyanaraman, R. Subramanian, *Anal. Chem.* 83 (2011) 9516.

- 1121 [162] G. Tan, Z. Lou, X. Dong, W. Li, W. Liao, Z. Zhu, Y. Chai, *Chromatographia* 74 (2011)  
1122 341.
- 1123 [163] G.P. Hooff, R.J.W. Meesters, J.J.A. van Kampen, N.A. van Huizen, B. Koch, A.F.Y. Al  
1124 Hadithy, T. van Gelder, A.D.M.E. Osterhaus, R.A. Gruters, T.M. Luider, *Anal. Bioanal.*  
1125 *Chem.* 400 (2011) 3473.
- 1126 [164] S. Strano-Rossi, L. Anzillotti, E. Castrignano, M. Felli, G. Serpelloni, R. Mollica, M.  
1127 Chiarotti, *Anal. Bioanal. Chem.* 401 (2011) 609.
- 1128 [165] W. Song, D. Pabbisetty, E.A. Groeber, R.C. Steenwyk, D.M. Fast, *J. Pharm. Biomed.*  
1129 *Anal.* 50 (2009) 491.
- 1130 [166] H. Trufelli, P. Palma, G. Famiglini, A. Cappiello, *Mass Spectrom. Rev.* 30 (2011) 491.
- 1131 [167] K.A. Hanold, S.M. Fischer, P.H. Cormia, C.E. Miller, J.A. Syage, *Anal. Chem.* 76 (2004)  
1132 2842.
- 1133 [168] J.F. Brouwers, *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids* 1811 (2011) 763.
- 1134 [169] O.J. Pozo, J.V. Sancho, M. Ibanez, F. Hernandez, W.M.A. Niessen, *TrAC Trends Anal.*  
1135 *Chem.* 25 (2006) 1030.
- 1136 [170] A. Kaufmann, P. Butcher, *Rapid Commun. Mass Spectrom.* 20 (2006) 3566.
- 1137 [171] A. Schurmann, V. Dvorak, C. Cruzler, P. Butcher, A. Kaufmann, *Rapid Commun. Mass*  
1138 *Spectrom.* 23 (2009) 1196.
- 1139 [172] E.N. Fung, Y.Q. Xia, A.F. Aubry, J.N. Zeng, T. Olah, M. Jemal, *J. Chromatogr. B: Anal.*  
1140 *Technol. Biomed. Life Sci.* 879 (2011) 2919.
- 1141 [173] J.P. Antignac, F. Courant, G. Pinel, E. Bichon, F. Monteau, C. Elliott, B. Le Bizec, *TrAC*  
1142 *Trends Anal. Chem.* 30 (2011) 292.
- 1143 [174] T. Moehring, M. Scigelova, C.S. Ejsing, D. Schwudke, A. Shevchenko, *Essential*  
1144 *Lipidomics Experiments using the LTQ Orbitrap Hybrid Mass Spectrometry, Application*  
1145 *Note: 367 (2007), Thermo Scientific.*
- 1146  
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1149 **Figure Captions**

1150  
1151 Figure 1. Schematic presentation of a synthetic route of surfactant-coated C<sub>12</sub>-MNPs. Reproduced  
1152 from ref. [98], with permission of Elsevier.

1153  
1154 Figure 2. Representative UHPLC-MS/MS chromatogram of glucocorticoids. (a) Standard with  
1155 cortisol, cortisone, 11-deoxycortisol, prednisolone and dexamethasone. (b) Dilute plasma  
1156 ultrafiltrate with cortisol. (c) Saliva sample with cortisol and cortisone. (d) Plasma ultrafiltrate  
1157 with cortisol. (e) Plasma sample from a patient collected at 09:00 after administration of 1 mg  
1158 dexamethasone at 23:00 the previous evening. (f) Urine from a patient on prednisolone treatment.  
1159 Reproduced from Ref. [142], with permission of Elsevier.

1160  
1161 Figure 3. Gradient UHPLC-MS/MS chromatograms of the (i) bromo-guanosine, (ii) labetalol,  
1162 (iii) reserpine and (iv) drug compound SB243213A test mix on several possible columns for  
1163 generic gradient UHPLC-MS bio-analysis. All columns were of 50 mm x 2.1 mm I.D.: (a) Luna  
1164 HST C18(2) 2.5 μm, (b) Luna PFP 3 μm, (c) Acquity BEH C18 1.7 μm (n=4 chromatograms  
1165 overlaid) and (d) Ascentis Express C18 2.7 μm (n=4 chromatograms overlaid). Reproduced from  
1166 Ref. [160], with permission of Elsevier.

1167  
1168 Figure 4. LTQ Orbitrap analysis of isobaric species PE 18:0/18:0 and PE O-16:1p/22:6. The time  
1169 needed for performing one scan at given resolution settings is highlighted in boxes. Reproduced  
1170 from Ref. [174], with permission of Thermo Scientific.

**Table 1. Use of on-line SPE procedures in bio-analytical applications.**

Target compounds	Sample	SPE Stationary phase	Elution Solvents	Analytical technique	Analytical features	Reference
<i>Pharmaceuticals and drugs</i>						
FTY720, FTY720-P	Blood	HySphere C18 HD 7mm	Dimethylhexylamine solution (A) and acetonitrile/isopropanol (80:20, v/v) (B)	LC-ESI-MS/MS	LOQs > 0.08 ng mL <sup>-1</sup>	[32]
Sirolimus and everolimus	Blood	Zorbax Extend-C18 (2.1 mm×12.5 mm, 5 μm)	Methanol (A) and water (B) both containing 0.1% formic acid in 2 mmol L <sup>-1</sup> ammonium formate	LC-ESI-MS/MS	LODs > 0.1 ng mL <sup>-1</sup> LOQs > 0.2 ng mL <sup>-1</sup>	[33]
Pharmaceutical compound and its metabolites	Plasma	Gemini C18 (2mm×10mm, 5 μm)	5mM ammonium formate and 0.2% formic acid in water/acetonitrile (80:20, v/v)	UHPLC-ESI-MS/MS	LOQs > 5 ng mL <sup>-1</sup>	[34]
Fluoxetine	Plasma	Kromasil C18 (1mm×5mm, 5 μm)	Acetonitrile and 0.05 mol L <sup>-1</sup> ammonium formate buffer (25:75, v/v)	Capillary LC-ESI-TOF-MS	LODs > 3 ng mL <sup>-1</sup> LOQs > 5 ng mL <sup>-1</sup>	[35]
Amlodipine	Plasma	HySphere C8 EC-SE (2 mm× 10)	After treatment with acetonitrile , 0.1% formic acid in water (A) and methanol (B).	LC-ESI-MS/MS	LOQ 0.10 ng mL <sup>-1</sup>	[36]
Fluconazole, itraconazole, posaconazole and voriconazole (antifungal drugs)	Plasma	Waters Oasis HLB column (2.1×20 mm, 25 μm)	Acetonitrile/water 80/20 (A), 2 mM ammonium formate (B)	LC-MS/MS	LOD 2–10 ng mL <sup>-1</sup>	[37]
Pharmaceuticals	Plasma	Oasis® HLB cartridge column (2cmL×2.1mmID, 25μm)	0.1% ammonium acetate (A) and 0.1% methylamine in methanol (B)	LC-MS/MS	LOD 19-100 pg mL <sup>-1</sup>	[38]
Pharmaceutical Remoxipride (dopamine D2 receptor antagonist)	Plasma and Brain homogenate and dialysate	Oasis@weak cationic exchange (WCX) cartridges	Acetonitrile with 16mM acetic acid (7:3, v/v, 0.1% TFA, pH 2).	LC-MS/MS	LOQ 0.5 ng mL <sup>-1</sup> (plasma)  LOQ 0.25-1.8 ng mL <sup>-1</sup> ( Brain homogenate and dialysate)	[39]
Indacaterol	Serum	Oasis MCX, (1mm× 10 mm)	After acidification, centrifugation, 0.03% ammonia (A) and methanol (B). Vinj 150 μl.	LC-ESI-MS/MS	LOQ 10 ng L <sup>-1</sup>	[40]
Tilidine, nortilidine, and bisnortilidine	Serum	OASIS WCX (10mm×1mm)	0.2% formic acid (A) and methanol (B)	LC-ESI-MS/MS	LOQs > 1 ng mL <sup>-1</sup>	[41]
Forty-two therapeutic drugs and drugs of abuse	Serum	SPE Strata X-CW (2mm×20mm, 25 μm)	0.5% HCOOH in water (A) and 0.5% HCOOH in acetonitrile (B) (90/10, v/v)	UHPLC-ESI-MS/MS	LODs > 0.20 ng mL <sup>-1</sup> LOQs > 0.66 ng mL <sup>-1</sup>	[42]
11-nor-Δ <sup>9</sup> -tetrahydrocannabinol-9-carboxylic acid	Serum	HySphere C8	0.1% formic acid (A) and acetonitrile (B)	LC-ESI-MS/MS	LODs > 0.25 ng mL <sup>-1</sup> LOQs > 5 ng mL <sup>-1</sup>	[43]
<i>Peptides</i>						
Sifuvirtide (anti-HIV peptide)	Plasma	C18 SPE column (50mm×2.1mmi.d., 2μmfrits)	0.2% formic acid in water (A), acetonitril/methanol (1:1) (B) (70:30)	LC-MS/MS	6.1 ng mL <sup>-1</sup>	[44]
Aggrecan fragments (peptide)	Urine and Human sinovial fluid	home-prepared immunoaffinity columns and macro peptide-trapping column (Michrom Bioresources, Auburn, CA, USA)	0.1% formic acid in water (A) and 5% acetonitrile in water (B)	LC-MS/MS	LOD 2.5 pg mL <sup>-1</sup> (Urine) LOD 10 pg mL <sup>-1</sup> (human synovial fluid)	[45]
<i>Personal care products</i>						
2-ethylhexyl	Human urine	HySphere C18 HD	0.2% formic acid in water (A) and 0.2% formic	LC-ESI-MS/MS	LODs > 0.1 ng	[46]



4-(N,N-dimethylamino)benzoate		(10mm×2mm, 7µm)	acid methanol/ acetonitrile (1/1, v/v) (B)	LC-ESI-TOF/MS	LOQs > 0.3 ng	
<i>Endogenous compounds</i>						
Tryptophan, kynurenine and 3-hydroxykynurenine	Plasma	Isolute PRS (propylsulphonic acid based strong cation exchange) (10mm×1mm)	50 mM ammonium formate (pH 3)	LC-ESI-MS/MS	LODs > 1 nmol L <sup>-1</sup> LOQs > 23 nmol L <sup>-1</sup>	[47]
Ethinylestradiol	Plasma	Hysphere C18 HD 7µm	Methanol/water (75/25, v/v)	LC-APPI-MS/MS	LODs > 0.08 pg mL <sup>-1</sup> LOQs > 5 pg mL <sup>-1</sup>	[48]
8-oxo-7,8-dihydro-2'-deoxyguanosine	Plasma, urine and saliva	Inertsil ODS-3 column (50 mm×4.6 mm, 5 µm)	50 mL L <sup>-1</sup> methanol containing 1 mL L <sup>-1</sup> formic acid (A) and 500 mL L <sup>-1</sup> methanol containing 1 mL L <sup>-1</sup> formic acid (B)	LC-ESI-MS/MS	LODs > 2 fmol	[49]
Eicosanoid inflammation biomarkers	Serum	HySphere C18 (EC) (10mm×2.0mm, 8 µm)	methanol/water/ acetonitrile/acetic acid (76/22/2/0.02, v/v)	LC-ESI-MS/MS	LODs > 0.09 pg mL <sup>-1</sup> ; LOQs > 3 pg mL <sup>-1</sup>	[50]
Serotonin	Serum	Oasis WCX (10 mm×1 mm)	100 mM ammonium formate (pH 3) (A) and acetonitrile (B)	LC-ESI-MS/MS	LOQs > 0.9 nmol mL <sup>-1</sup>	[51]
25-Hydroxyvitamin D	Serum	Waters 5 µm X-Terra (2.1 mm×20 mm C18)	(98:2) methanol:water 0.1% formic acid and 2 mM ammonium acetate(A), water with 0.1% formic acid and 2 mM ammonium acetate (B)	LC-MS/MS	LOQ 3 nmol L <sup>-1</sup>	[52]
Cortisol, cortisone and metabolites	Urine	POROS R1/20 (2.1mm×30 mm, 20 µm particle size)	methanol/acetonitrile (50:50, v/v) (A), and water and methanol (50:50, v/v) (B), both containing 0.1% formic acid	LC-ESI-MS/MS	--	[53]
Methylmalonic acid	Urine	Waters Oasis HLB (2.1×20 mm, 25 µm)	1 g L <sup>-1</sup> formic acid-acetonitrile/10 mM ammonium formate 60:40 (v/v)	LC-ESI-MS/MS	--	[54]
Phthalate metabolites	Urine	Merck C18 trap cartridge (2.0 x 55-mm, 3µm, ,)	0.001% formic acid in H <sub>2</sub> O (A), metanol (B)	LC-MS/MS	LOD 0.2-2 ng mL <sup>-1</sup>	[55]
Cortisol and cortisone	Human saliva	HySphere™ C18 HD (2 mm 10mm, 7 µm SPE cartridge)	0.1% (v/v) formic acid in water (A), 0.1% (v/v) formic acid in methanol (B) (50:50)	LC-MS/MS	LOQ Cotisol 0.75 nM Cortisone 0.50 nM	[56]
8-iso-PGF <sub>2</sub> α (indicator of lipid peroxidation)	Exhaled breath condensate	C18 Inertsil ODS (33.3mm×4.6mm, 5µm) column	1% acetonitrile, v/v with 0.1% formic acid(A), 90% acetonitrile, v/v with 0.1% formic acid (B) (85:15)	LC-MS/MS	LOD 1 pg mL <sup>-1</sup>	[57]
<i>Pollutants</i>						
Chlorpyrifos and cypermethrin	Plasma	Hypersil GOLD C8 (20mm×2.1 mm, 1.9µm)	20 mM ammonium acetate aqueous solution and methanol (10:90, v/v)	LC-ESI-MS/MS	LODs > 0.01 ppb	[58]
Perfluorinated compounds	Plasma	Poros HQ (2.1mm×30mm, 10µm)	0.01% NH <sub>4</sub> OH solution in 5mM ammonium acetate (A) and 0.01% NH <sub>4</sub> OH solution in acetonitrile (B)	UHPLC-ESI-MS/MS	LODs > 3 ng L <sup>-1</sup> LOQs > 10 ng L <sup>-1</sup> MDLs > 9 ng L <sup>-1</sup>	[59]
Polyfluorinated compounds	Serum	Polaris C18 HD (2mm 10mm)	20mM ammonium acetate (pH 4) in water (A), acetonitrile (B)	LC-MS/MS	LOD 0.1- 0.2 ng mL <sup>-1</sup>	[60]
Polyfluorinated compounds	Serum and human breast milk	Oasis® HLB, (2.1mm×20mm 25µm)	38% 2mM ammonium acetate buffer adjusted to pH 5 with acetic acid (A), 62% methanol (B)	LC-MS/MS	0.1 to 0.4µg L <sup>-1</sup> (serum) 0.02 to 0.15µg L <sup>-1</sup> (human breast milk)	[61]
Intermediates of the tricarboxylic acid cycle	Pig and mouse heart tissue	Phenomenex C8 SPE cartridge (4 mm x 2 mm, 5 µm)	98% water, 2% acetonitrile and 0.1% formic acid (A), (98% acetonitrile, 2% water and 0.1% formic acid (B)	LC-ESI-MS	LOD 12-1000 nM	[62]



**Table 2. Use of turbulent flow chromatography in bioanalysis. Selected references from 2009-up-to-date.**

Target compounds	Class	Matrix	TFC Column	Flow-rate Injection Volume	Detection	LOQ	Reference
Sirolimus and everolimus	Immunosuppressant	Whole blood	50 x 0.5 mm, 50 $\mu$ m Cyclone (Thermo Fisher Scientific)	2.0 mL min <sup>-1</sup> 50 $\mu$ L	APCI-MS/MS	0.5 ng mL <sup>-1</sup>	[106]
453 Drugs	General - toxicological screening	Urine and serum	50 x 0.5 mm, 60 $\mu$ m Cyclone and C18 XL (Thermo Fisher Scientific)	2.0 mL min <sup>-1</sup> 100 $\mu$ L	APCI-MS <sup>a</sup>	-	[107]
Perfluorinated compounds	Perfluorinated compounds	Human hair and urine	50 x 0.5 mm, 60 $\mu$ m Cyclone and C18 XL (Thermo Fisher Scientific)	1.5 mL min <sup>-1</sup> 20 $\mu$ L	ESI-MS/MS	0.06-13.34 ng g <sup>-1</sup>	[108]
25-OH D2/D3	Vitamins	Serum	Cyclone P 50 x 1.0 mm (Thermo Fisher Scientific)	4.0 mL min <sup>-1</sup> 100 $\mu$ L	APCI-MS/MS	4.6 nmol L <sup>-1</sup> (25-OHD3) 3.0 nmol L <sup>-1</sup> (25-OHD2)	[109]
-	Metabonomic studies	Plasma	50 x 0.5 mm, 50 $\mu$ m Cyclone (Thermo Fisher Scientific)	1.25 mL min <sup>-1</sup> 10 $\mu$ L	ESI-MS	-	[104]
Busulfan	Alkylating antineoplastic agent	Plasma and serum	Cyclone P 50 x 0.5 mm (Thermo Fisher Scientific)	1.5 mL min <sup>-1</sup> 50 $\mu$ L	ESI-MS/MS	0.15 $\mu$ mol L <sup>-1</sup>	[110]
9 TKIs and metabolites	Tyrosine kinase inhibitors	Plasma and serum	50 x 0.5 mm, 50 $\mu$ m Cyclone (Thermo Fisher Scientific)	2.0 mL min <sup>-1</sup> 100 $\mu$ L	APCI-MS/MS	1-10 ng mL <sup>-1</sup> <sup>a</sup>	[111]
Creatinine	Creatinine	Serum	50 x 0.5 mm Cyclone MCX (Thermo Fisher Scientific)	1.5 mL min <sup>-1</sup> 20 $\mu$ L	ESI-MS/MS	0.20 mg dL <sup>-1</sup>	[112]
-	Pharmacokinetics	Plasma and hepatocytes	50 x 0.5 mm, 60 $\mu$ m Cyclone (Thermo Fisher Scientific)	1.25 mL.min <sup>-1</sup> 20 $\mu$ L	ESI-MS/MS	-	[113]
356 Drugs and metabolites	General - toxicological screening	Urine	50 x 0.5 mm, 60 $\mu$ m Cyclone and C18 XL (Thermo Fisher Scientific)	2.0 mL min <sup>-1</sup> 100 $\mu$ L	APCI-MS <sup>a</sup>	<sup>b</sup> 10 ng L <sup>-1</sup> (60%) 100 ng L <sup>-1</sup> (90%) 1000 ng L <sup>-1</sup> (100%)	[114]
Amitriptyline Desipramine Imipramine Nortriptyline	Tricyclic Antidepressants	Serum	Cyclone P 50 x 0.5 mm (Thermo Fisher Scientific)	2.0 mL min <sup>-1</sup> 10 $\mu$ L	ESI-MS/MS	6-18 ng mL <sup>-1</sup>	[115]
Metanephrine and Nometanephrine	Metanephrines	Plasma	50 x 0.5 mm MCX-2 (Thermo Fisher Scientific)	2.0 mL min <sup>-1</sup> 100 $\mu$ L	ESI-MS/MS	6.3-12.6 pg mL <sup>-1</sup>	[116]
Verticine Verticinone Isoverticine	Drugs	Plasma	20 x 2.1 mm, 25 $\mu$ m Oasis HLB (Waters)	4.0 mL min <sup>-1</sup> 50 $\mu$ L	ESI-MS	0.12-0.595 ng mL <sup>-1</sup>	[117]

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19 drugs and metabolites	Drugs	Urine	50 x 0.5 mm Cyclone P and Cyclone P (Thermo Fisher Scientific)	2.0 mL.min <sup>-1</sup> 100 µL	ESI-MS/MS	5-25 ng mL <sup>-1</sup>	[118]
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<sup>a</sup> Reported as limit of accurate measurement (signal at least five times the SD of the background noise)

<sup>b</sup> Reported as limited of identification

**Table 3. UHPLC bio-analytical applications using sub-2 µm particle size packed columns.**

Target compounds	Sample matrix	Column / Stationary phase/Temperature	Mobile phase / Flow-rate	Mass spectrometry	Analysis time	Reference
JAK2 inhibitor CYT387	Plasma	Acquity UPLC BEH C18 (30 mm x 2.1 mm, 1.7 µm) 40 °C	Gradient elution: A) 0.005% formic acid solution in water B) 0.05 % formic acid solution in methanol 0.6 mL min <sup>-1</sup>	HESI(+) Triple quadrupole SRM acquisition mode	1.3 min	[133]
Ketoconazole	Plasma	Acquity UPLC BEH C18 (50 mm x 2.1 mm, 1.7 µm)	Isocratic elution: Water:acetonitrile 44:56 (v/v) with 0.1% ammonium hydroxide and 10 mM ammonium bicarbonate 1.0 mL min <sup>-1</sup>	ESI(+) Triple quadrupole SRM acquisition mode	20 s	[134]
Vitamin D metabolites	Plasma	Acquity UPLC BEH C18 (50 mm x 2.1 mm, 1.7 µm)	Gradient elution: A) 0.1% formic acid in water (5 mM methylamine) B) 0.1% formic acid in methanol 0.3 mL min <sup>-1</sup>	ESI(+) Triple quadrupole-linear ion trap SRM acquisition mode	2.2 min	[135]
Anticancer PR-104 and metabolites	Plasma	Zorbax Eclipse XDB-C18 (50 mm x 2.1 mm, 1.8 µm)	Gradient elution: A) 0.01% formic acid in water B) acetonitrile 0.5 mL min <sup>-1</sup>	Positive ESI-APCI combined mode Triple quadrupole SRM acquisition mode	3.1 min	[136]
Small-molecule complements in Biological system	Plasma	Acquity UPLC BEH C18 (50 mm x 2.1 mm, 1.7 µm) 40 °C	Gradient elution: <i>Extracts reconstituted in formic acid</i> A) 0.1% formic acid in water B) 0.1% formic acid in methanol <i>Extracts reconstituted in ammonium bicarbonate</i> A) 6.5 mM ammonium bicarbonate in water, pH 8 B) 6.5 mM ammonium bicarbonate in methanol:water 95:5 (v/v) 0.35 mL min <sup>-1</sup>	ESI(+) and ESI(-) Linear ion trap MS SRM acquisition mode  <i>Accurate mass measurements</i> Hybrid LTQ-FTICR M (Resolving power 50,000 FWHM)	8 min	[137]
Pravastatin Pravastatin lactone	Plasma Urine	Acquity UPLC BEH C18 (50 mm x 2.1 mm, 1.7 µm) 35 °C	Gradient elution: A) 1 mM ammonium acetate pH 4.0 B) acetonitrile 0.2 mL min <sup>-1</sup>	ESI polarity switching mode Triple quadrupole SRM acquisition mode	2 min	[138]
Tectorigenin, irigenin and irisflorethin	Plasma Urine	Zorbax SB-C18 (50 mm x 2.1 mm, 1.8 µm)	Gradient elution: A) 0.1% formic acid in water B) acetonitrile 0.4 mL min <sup>-1</sup>	ESI(-) and ESI(+) Triple quadrupole SRM acquisition mode	6 min	[139]
Octreotide	Plasma	Acquity UPLC BEH C18 (100 mm x 2.1 mm, 1.7 µm)	Gradient elution: A) 0.1% formic acid in water B) 0.1% formic acid in acetonitrile 0.25-0.3 mL min <sup>-1</sup>	ESI(+) Triple quadrupole SRM acquisition mode	5 min	[66]
Clopidogrel active metabolite isomers	Plasma	Shim-pack XR-ODS II (75 mm x 2.0 mm, 2.2 µm)	Gradient elution: A) 2 mM ammonium acetate-0.2% formic acid in water B) 2 mM ammonium acetate-0.2% formic acid in acetonitrile 0.5 mL min <sup>-1</sup>	ESI(+) Triple quadrupole SRM acquisition mode	7 min	[140]
Eicosapentaenoic acid	Plasma	Acquity UPLC BEH C18	Gradient elution:	ESI(-)	1.3 min	[141]

and docosahexenoic acid		(50 mm x 2.1 mm, 1.7 $\mu$ m) 60 °C	A) 2 mM ammonium acetate pH 4 B) acetonitrile 1 mL min <sup>-1</sup>	Triple quadrupole SRM acquisition mode		
Cortisol, cortisone, prednisolone, dexamethasone and 11-deoxycortisol	Plasma, plasma ultrafiltrate, urine and saliva	Acquity UPLC BEH C18 (50 mm x 2.1 mm, 1.7 $\mu$ m)	Gradient elution: A) 2 mM ammonium acetate – 0.1 % formic acid in water B) 2 mM ammonium acetate – 0.1% formic acid in methanol 0.4 mL min <sup>-1</sup>	ESI(+) Triple quadrupole SRM acquisition mode	2.5 min	[142]
Nitrofurantoin metabolites	Plasma	Acquity UPLC BEH C18 (100 mm x 2.1 mm, 1.7 $\mu$ m) 65 °C	Gradient elution: A) 0.5 mM ammonium acetate in water B) methanol 0.5 mL min <sup>-1</sup>	ESI(+) Triple quadrupole SRM acquisition mode	3 min	[143]
Testosterone and testosterone enanthate	Plasma	Hypersil Gold C18 (50 mm x 2.1 mm, 1.9 $\mu$ m) 45 °C	Gradient elution: A) 2 mM ammonium formate in water B) methanol 0.5 mL min <sup>-1</sup>	HESI(+) Triple quadrupole SRM acquisition mode	4.5 min	[144]
Anandamide and related acylethanolamides	Human seminal plasma	Acquity UPLC BEH C18 (50 mm x 2.1 mm, 1.7 $\mu$ m)	Gradient elution: A) 2 mM ammonium formate containing 0.1% formic acid and 5% acetonitrile B) 0.1% formic acid in acetonitrile 0.7 mL min <sup>-1</sup>	ESI(+) Triple quadrupole SRM acquisition mode	3 min	[145]
Diuretic and stimulant compounds	Urine	Zorbax SB-C8 (50 mm x 2.1 mm, 1.8 $\mu$ m)	Gradient elution: A) 1 mM ammonium acetate – 0.001% acetic acid in water B) 1 mM ammonium acetate - 0.001% acetic acid in methanol 0.3 mL min <sup>-1</sup>	ESI polarity switching mode HRMS Orbitrap	8 min	[146]
Anabolic steroids	Urine	Acquity UPLC BEH C18 (100 mm x 2.1 mm, 1.7 $\mu$ m)	Gradient elution: A) 5 mM ammonium formate pH 3 in water B) 0.1% formic acid in acetonitrile 0.4 mL min <sup>-1</sup>	HESI(+) Triple quadrupole SRM acquisition mode	8 min	[147]
Cardiovascular drugs, polyphenols and metabolites	Urine	Hypersil Gold C18 (100 mm x 2.1 mm, 1.9 $\mu$ m) 25 °C	Gradient elution: A) 0.1% formic acid in water B) acetonitrile 0.65-0.7 mL min <sup>-1</sup>	ESI(+) and ESI(-) Triple quadrupole-linear ion trap SRM acquisition mode	8 min	[148]
Therapeutic drugs and drugs of abuse	Urine	Zorbax Eclipse XDB-C18 (50 mm x 4.6 mm, 1.8 $\mu$ m) 55 °C	Gradient elution: A) 0.5% formic acid in water B) 0.5% formic acid in acetonitrile 0.9 mL min <sup>-1</sup>	ESI(+) QTrap MS SRM acquisition mode	6 min	[42]
Thyroid hormones	Urine	Acquity UPLC HSS T3 (high strength silica particles) (100 mm x 2.1 mm, 1.8 $\mu$ m) 25 °C	Gradient elution: A) 0.1% formic acid in water B) 0.1% formic acid in methanol 0.3 mL min <sup>-1</sup>	HESI(+) Triple quadrupole SRM acquisition mode	6.5 min	[149]
Rifampicin isonicotinyl hydrazone (HYD)	Plasma, urine and faeces	Zorbax Eclipse C18 (50 mm x 4.6 mm, 1.8 $\mu$ m)	Gradient elution: A) 10 mM ammonium acetate in water B) acetonitrile 0.5 mL min <sup>-1</sup>	ESI(+) Linear ion trap MS SRM acquisition mode	5 min	[150]
2-tert-butylprimaquine (anti-	Plasma	Hypersil Gold C18 (50 mm x 2.1 mm, 1.9 $\mu$ m)	Gradient elution: A) 20 mM ammonium acetate	ESI(+) Linear ion trap MS	4 min	[151]

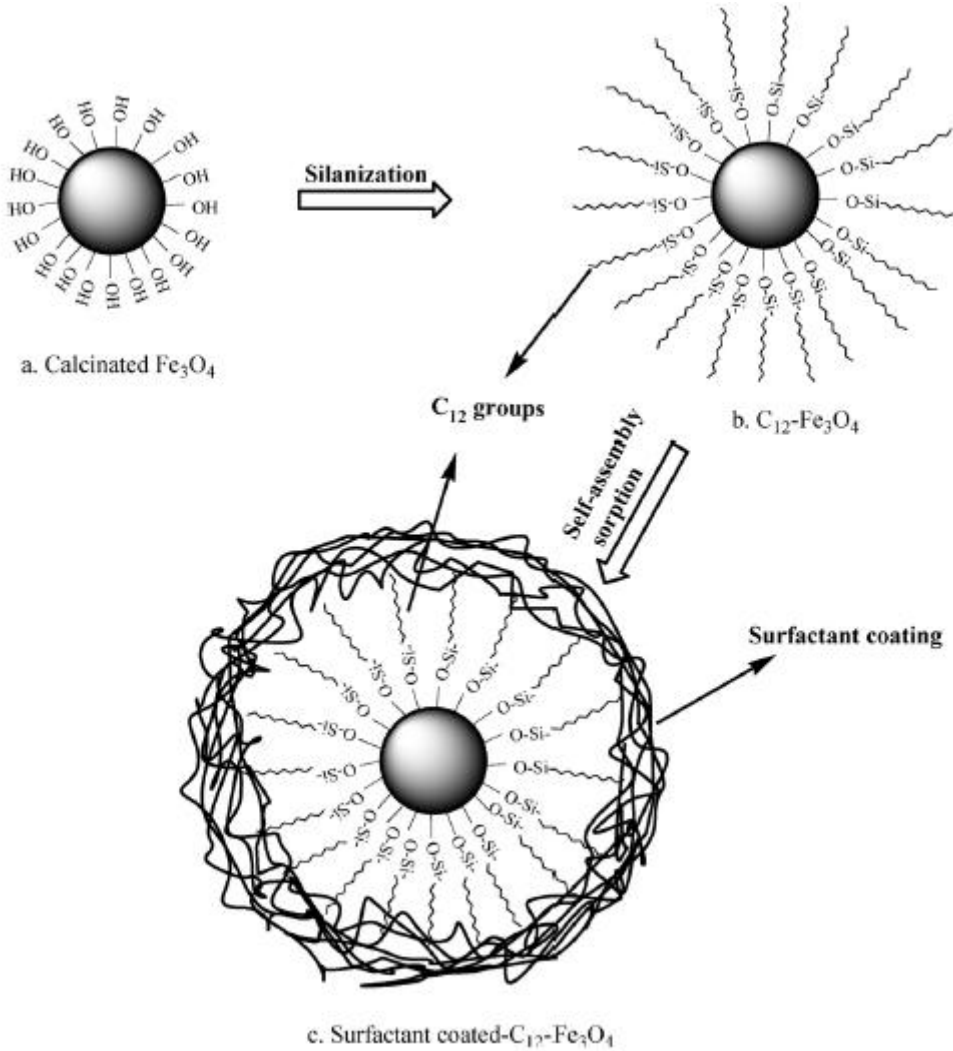
malarial compound)			B) acetonitrile 0.45 mL min <sup>-1</sup>	SRM acquisition mode		
Doxorubicin and daunorubicin	Liver tumors	Shimadzu Shim-Pack ODS (50 mm x 2.0 mm, 1.6 µm)	Gradient elution: A) 5 mM ammonium acetate (pH 3.5) in water B) acetonitrile 0.5 mL min <sup>-1</sup>	ESI(+) Triple quadrupole SRM acquisition mode	1.1 min	[152]
Anti-tumoral alkyl lysophospholipid edelfosine	Plasma, tissue, tumor and lipid nanoparticulate systems	Acquity UPLC BEH C18 (50 mm x 2.1 mm, 1.7 µm) 50 °C	Isocratic elution: 1% formic acid aqueous solution:methanol 5:95 (v/v) 0.5 mL min <sup>-1</sup>	ESI(+) Triple quadrupole SRM acquisition mode	4 min	[153]
Neurotransmitters	Brain tissue	Acquity UPLC BEH C18 (50 mm x 2.1 mm, 1.7 µm)	Gradient elution: A) 0.05% formic acid and 1 mM heptafluorobutyric (HFBA) acid in water B) methanol 0.2 mL min <sup>-1</sup>	ESI(+) Triple quadrupole SRM acquisition mode	4 min	[154]
Atorvastatin and its metabolites	Serum	Acquity UPLC BEH C18 (100 mm x 2.1 mm, 1.7 µm) 35 °C	Gradient elution: A) 0.5 mM ammonium acetate (pH 4.0) in water B) acetonitrile 0.25 mL min <sup>-1</sup>	ESI(+) Triple quadrupole SRM acquisition mode	3.5 min	[155]
Emodin	Plasma from digestive segments	Acquity UPLC BEH C18 (100 mm x 2.1 mm, 1.7 µm) 35 °C	Gradient elution: A) 0.1% formic acid in water B) methanol 0.3 mL min <sup>-1</sup>	ESI(-) Triple quadrupole SRM acquisition mode	4 min	[156]
Perfluorochemicals	Plasma and serum	Zorbax Eclipse XDB-C18 (50 mm x 4.6 mm, 1.8 µm) 55 °C	Gradient elution: A) 0.01% NH <sub>4</sub> OH, 5 mM ammonium acetate in water B) 0.01% NH <sub>4</sub> OH in acetonitrile 1 mL min <sup>-1</sup>	ESI(-) QTrap MS SRM acquisition mode	5 min	[59]
Chlorpyrifos and cypermethrin	Cord blood plasma	Hypersil Gold C18 (50 mm x 2.1 mm, 1.9 µm)	Gradient elution: A) 20 mM ammonium acetate in water B) methanol 0.3 mL min <sup>-1</sup>	HESI(+) Triple quadrupole SRM acquisition mode	5 min	[58]
Prohibited drugs in doping control	Dried blood spots	Hypersil Gold C18 (50 mm x 2.1 mm, 1.9 µm)	Gradient elution: A) 0.2% formic acid in water B) acetonitrile 0.2 mL min <sup>-1</sup>	HESI(+) and HESI(-) Quadrupole-Orbitrap Full scan and all-ion fragmentation full scan acquisition modes	11 min	[157]

**Table 4. Bio-analytical applications using porous shell column technology.**

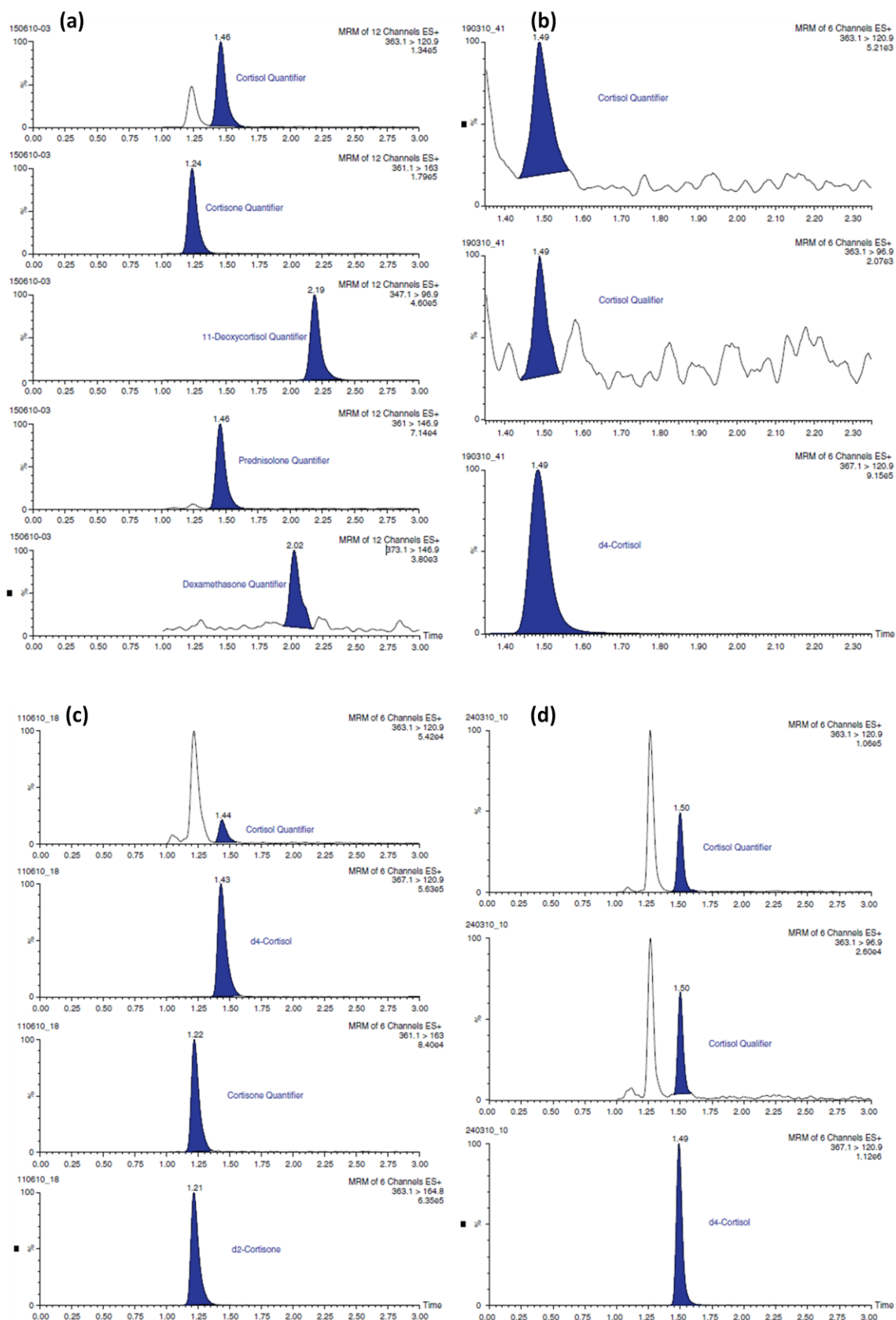
Target compounds	Sample matrix	Column / Stationary phase/Temperature	Mobile phase / Flow-rate	Mass spectrometry	Analysis time	Reference
Glutathione-trapped reactive metabolites	Plasma	Kinetex C18 (100 mm x 2.1 mm, 2.6 $\mu\text{m}$ )	Gradient elution: A) 0.1 % formic acid in water B) 0.1% formic acid in acetonitrile 0.4-0.6 mL min <sup>-1</sup>	HESI(+) and HESI(-) Linear ion trap-Orbitrap HRMS SIM acquisition mode and FTMS or ITMS data dependent scan of MS <sup>2</sup> spectra	10 min	[161]
Isoliquiritigenin metabolites	Urine	Agilent Poroshell EC-C18 (100 mm x 2.1 mm, 2.7 $\mu\text{m}$ ) 40 °C	Gradient elution: A) 0.1% formic acid in water B) 0.1% formic acid in acetonitrile 0.4 mL min <sup>-1</sup>	ESI(+) and ESI(-) TOF MS	12 min	[162]
Osetamivir and osetamivircarboxylate	Dried blood spots	Ascentix Express C18 (100 mm x 2.1 mm, 2.7 $\mu\text{m}$ ) 30 °C	Gradient elution: A) 0.1% formic acid in water B) 0.1% formic acid in methanol 0.35 mL min <sup>-1</sup>	ESI(+) QTrap MS SRM acquisition mode	3.5 min	[163]
Bromo-guanosine, labetalol, reserpine and SB243213A (drug compound)	Plasma	Ascentix Express C18 (50 mm x 2.1 mm, 2.7 $\mu\text{m}$ ) 40 °C	Gradient elution: A) 0.1% formic acid in water B) 0.1% formic acid in acetonitrile 1.1 mL min <sup>-1</sup>	ESI(+) Triple quadrupole SRM acquisition mode	0.8 min	[160]
Drugs of abuse	Oral fluid	Kinetex C18 (100 mm x 2.1 mm, 2.6 $\mu\text{m}$ ) 40 °C	Gradient elution: A) 0.05% formic acid and 5 mM ammonium formate in water B) 0.1% formic acid in methanol:acetonitrile 1:1 (v/v) 0.5 mL min <sup>-1</sup>	ESI(+) Triple quadrupole SRM acquisition mode	8 min	[164]
Imipramine and desipramine antidepressants	Plasma	Halo fused-core C18 (30 mm x 2.1 mm, 2.7 $\mu\text{m}$ )	Gradient elution: A) 10 mM ammonium acetate and 0.1% NH <sub>4</sub> OH in water (pH 8.5) B) 10 mM ammonium acetate, 0.1% NH <sub>4</sub> OH and 0.2% (morpholine or triethylamine) in acetonitrile 0.4 mL min <sup>-1</sup>	ESI(+) Triple quadrupole SRM acquisition mode	2.5 min	[165]



Figure 1



**Figure 2**



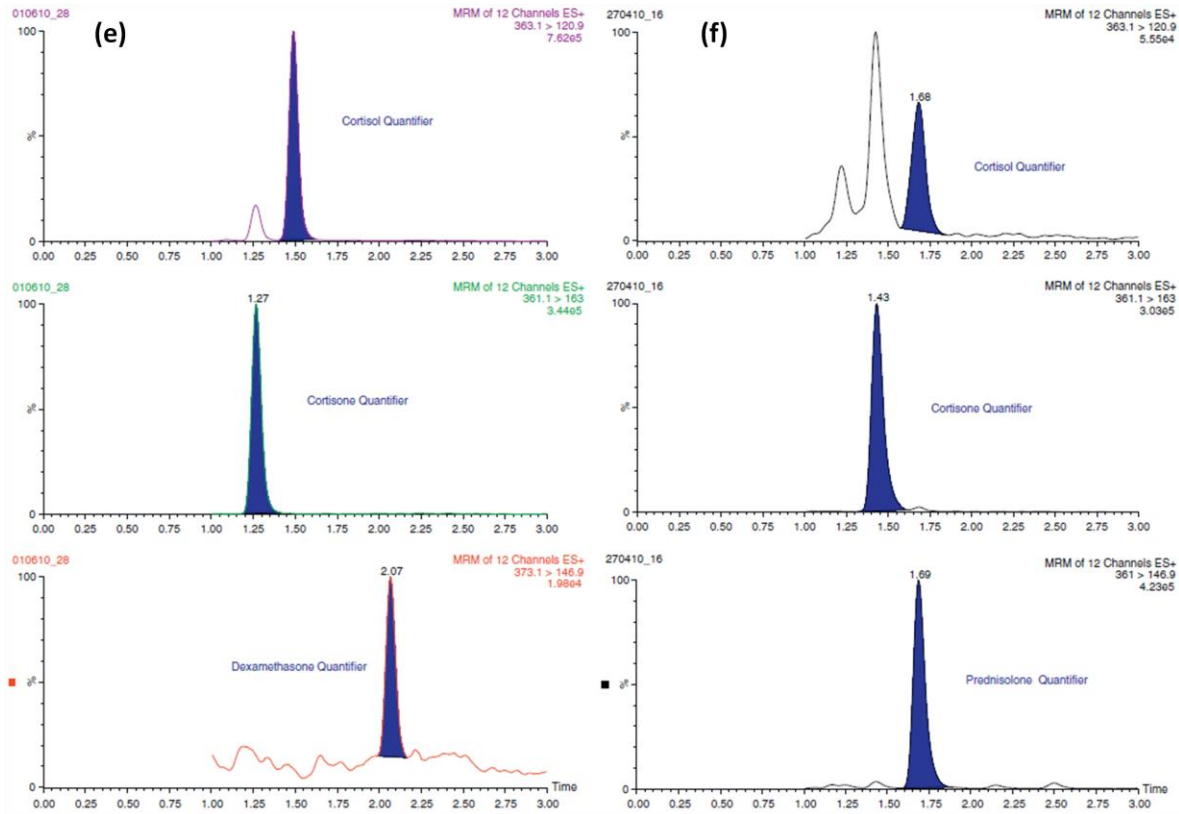
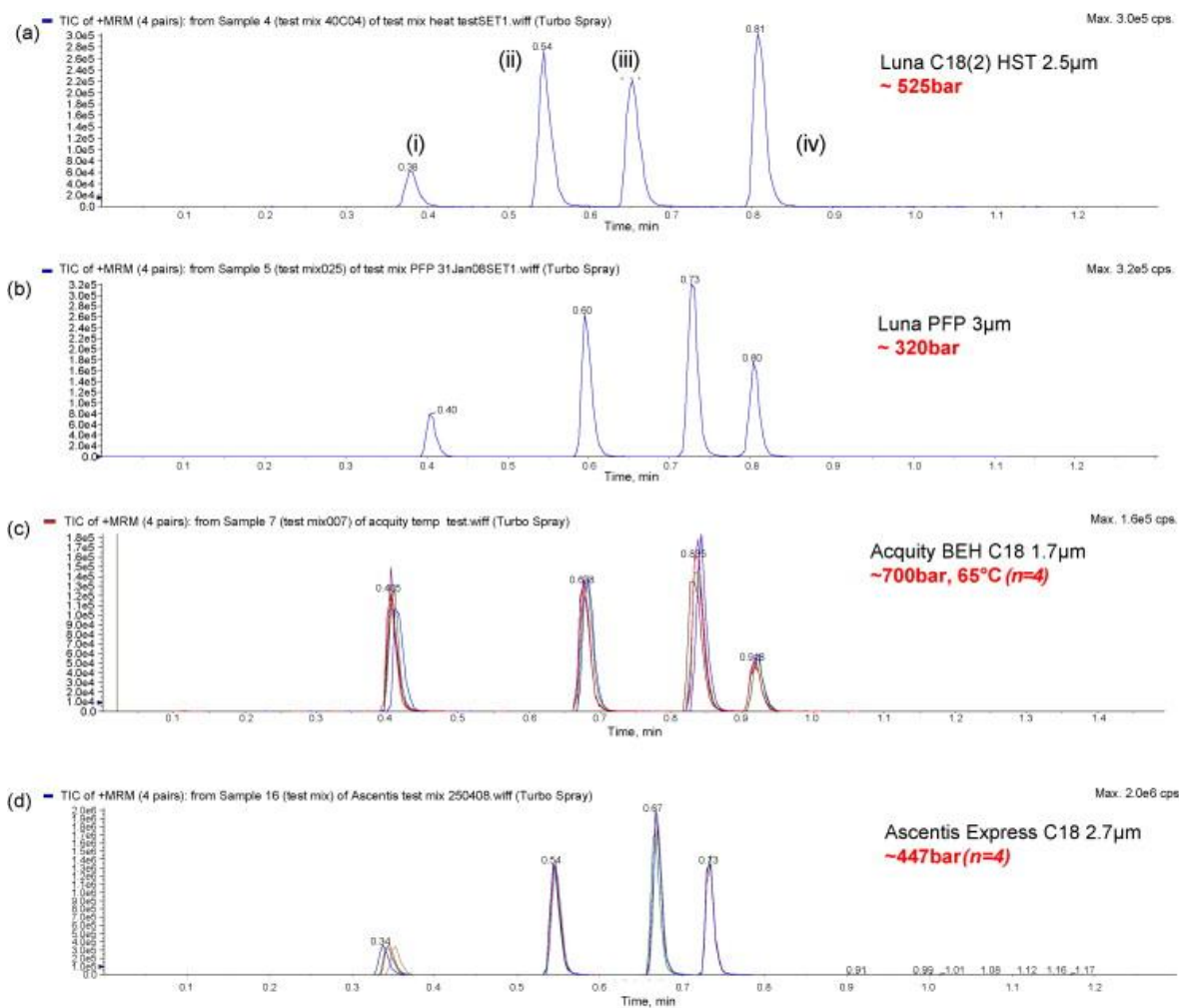


Figure 3



**Figure 4**

